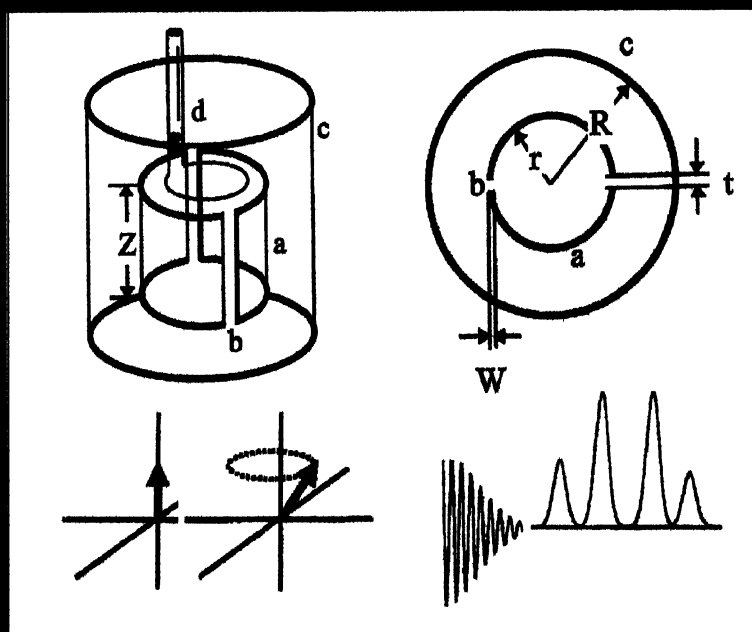


MAGNETIC RESONANCE



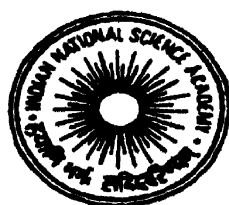
Edited by
P T Manoharan



INDIAN NATIONAL SCIENCE ACADEMY
NEW DELHI

MAGNETIC RESONANCE

Guest Editor
Professor P T Manoharan, FNA



INDIAN NATIONAL SCIENCE ACADEMY
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INSA EDITOR'S NOTE

Applications of Magnetic Resonance have had a phenomenal growth since its discovery half a century ago. It has an ever-growing potential as a subject of research by itself as well as a diagnostic tool for investigations in different fields of research from agriculture to medicine, molecular biology to material science, etc.

The present volume is an outcome of consistent effort of experts in the area who presented their deliberations in the INSA Seminar held on 17th March, 1997 in the Academy premises. We hope, the articles prepared by the speakers based on their deliberations in the above Seminar presented in this Book would be of lasting value.

We are grateful to the authors for their important contributions in generating this volume. We owe our indebtedness to Prof P T Manoharan, FNA who successfully accomplished the herculean task of convening the seminar, collecting the articles from respective authors and meticulously editing them so as to conform to INSA style of publications. Lastly, we appreciate the able editorial assistance rendered by Shri A K Tagore and Shri M Ranganathan.

December 15, 1999

S K Malik
Editor of Publications, INSA

PREFACE

No subject of recent discovery has been as exciting, useful, omnipresent and omnipotent as magnetic resonance. Since its discovery in 1945 in the US and USSR by Bloch and Purcell and Zavoisky, it has grown leaps and bounds, be it in its internal growth in the basic form of EPR, NMR and NQR to their value-added techniques such as FT NMR, FT EPR, double and triple resonances, correlation spectroscopies or in its growing and demanding applications in physics, chemistry, biology, geology and above all medicines. This is what prompted us to conduct an INSA Seminar in 1997 so that we can show a flavour of this most powerful and elegant subject to the general fellowship and other scientists by presenting it in a semipopular orientation.

Magnetic resonance has provided every access to probe the structure of molecules, devise new materials and even choose medicine and drugs. Indian scientists from early fifties have been involved in the development and utility of this technique. Now, there are at least five centres of excellence for magnetic resonance in this country. So, we did not find it difficult to choose experts who can talk authoritatively on various aspects of magnetic resonance and its applications. Successful indeed, the seminar was! It was, however, sad that we could not bring it to print early enough.

Though a total of nine lectures were delivered in this one-day seminar, only seven could materialise in print. This issue, a product of the said seminar, focusses on magnetic resonance from its evolutionary aspect to the development of techniques in view of enormous progress in the field of electronics and computer. Also, one finds applications to biomolecules, pharmaceuticals, agriculture and superconductivity. I am extremely grateful to all the authors for having provided me with the manuscripts. It is sad that applications of EPR and its allied techniques have found less of an expression in this issue in view of my failure in receiving the manuscripts from the authors.

I put on record my sincere thanks to Dr N Chandrakumar of Central Leather Research Institute, Chennai for having helped me in editing the manuscripts and providing the cover page illustrations in the form of a collage. I am sure that the readers of this issue will find it useful and interesting.

P T Manoharan

Guest Editor

Special Volume on '*Magnetic Resonance*'

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EVOLUTION OF MAGNETIC RESONANCE SPECTROSCOPY

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A review of magnetic resonance spectroscopy from its beginning to the current developments is presented. The phenomenological spin-Hamiltonian of nuclear magnetic resonance, the methods and constraints of high resolution NMR, evolution of Fourier transform NMR spectroscopy are presented. A brief introduction to 2D-FT NMR methods like COSY, NOESY and EXSY is given. Magnetic resonance imaging by 2D and 3D-FT NMR spectroscopy is described. EPR spin-Hamiltonian, the advantage of loop-gap resonators over the conventional cavity resonators are presented. The modern trends in EPR spectroscopy, its applications to biological systems, recent developments in EPR spectroscopy with the availability of fast computers and sophisticated EPR components including FT EPR spectroscopy, electron spin echoes, electron spin echo envelop modulation, and 2D-FT EPR spectroscopy are discussed. Electron-Zeeman resolved EPR spectroscopy, EPR imaging technique and *in vivo* EPR spectroscopy, electron-nuclear double resonance, Mössbauer and nuclear quadrupole resonance spectroscopies also find an expression in this report.

Key Words : EPR; NMR; Spin Echo; FT EPR; FT NMR; ENDOR; MRI, ESEEM; EPR Imaging

1 Introduction

Magnetic resonance was an excitement to scientists when it was invented in 1940s that today finds growing applications in physics, chemistry, biology, geology and medical diagnosis. Numerous scientists contributed wonderful ideas for its development over the past five decades that resulted in a variety of magnetic resonance fields. The nuclear spin is dealt by nuclear magnetic resonance (NMR), nuclear quadrupole resonance (NQR), and Mössbauer spectroscopy and the electron spin by electron paramagnetic resonance (EPR) while electron nuclear double resonance (ENDOR) combines both NMR and EPR. The differences between the electron and nuclear spin environments, sensitivities and the differences in their instrumentation led to the independent growth of the above techniques.

The classical NMR experiments by Edward M Purcell¹ and Felix Bloch² were honoured by a Nobel prize in 1952. The basic theory of chemical shifts and indirect spin-spin couplings (J) was developed by Norman F. Ramsay *et al.*³ Richard R Ernst received a Nobel prize in 1992 for his contribution to Fourier Transform NMR spectroscopy.⁴ Magnetic resonance in general

provides easy handling for the testing of novel experiments to demonstrate concepts of quantum mechanics, quantum statistics while its applications are of great interest in physics, chemistry, biology and medicine.

The introduction of Fourier transform (FT) spectroscopy in NMR instrumentation in 1964,⁵ relieved it from poor sensitivity problems and paved a path for modern trends. A variety of pulse sequences were developed replacing most of the continuous wave (CW) techniques. Novel methods were invented that were not thought of before. The capabilities of NMR were extended to two and three dimensions⁶ transforming NMR into a molecular structure determination tool competing with X-ray crystallography. The Correlation spectroscopy (COSY), Nuclear Overhauser effect spectroscopy (NOESY) and Exchange spectroscopy (EXSY) in two and three dimensions based on coherence transfer extract information on chemical shifts of nuclei that are coupled *via* scalar or dipolar coupling. Such experiments enable identification of the topology of homo- and heteronuclear coupling networks; elucidation of the connectivity of transitions in the energy-level diagram; and determination of the magnitudes and

relative signs of scalar and dipolar couplings. On the other hand, magnetic resonance imaging (MRI) that was originally demonstrated by Paul Lauterbur in 1973⁷, showed enormous impact on medical diagnosis for its ability to image human organisms non-invasively. Both the instrumentation and methodology of MRI had undergone several improvements and now MRI is exploring the possibility of imaging the functional behaviour of organisms^{8a,b}.

EPR spectroscopy after its discovery by Zavoisky⁹ in 1945 rapidly developed in parallel with NMR as it is a sensitive technique for the study of free radical chemistry and transition metal chemistry^{10,11}. The fact that EPR is thousands of times more sensitive to NMR, has resulted in a wide range of applications in physics, chemistry, biology, and mineralogy.

The development of EPR theory begins with the important contributions of Bloch² and Purcell¹², by incorporating Bethe's electrostatic crystal field theory¹³ and Van Vleck's theory of diamagnetism and paramagnetism and theory of moments¹⁴. Spin-Hamiltonian for zero-field splittings was presented by Abragam and Pryce¹⁵ and the molecular orbital approach to interpret *g*-factors was presented by Griffiths¹⁶, McGarvey¹⁷ and Orgel¹⁸. The development of CW EPR instrumentation was gradually evolved over a decade by optimization of cavity design, microwave components, development of homodyne and superheterodyne detection schemes including field modulation¹⁹.

EPR spectroscopy used CW mode operation for decades with great success. Though pulsed methods were introduced in EPR spectroscopy at the same time as in NMR a large part of EPR community was discouraged by the expensive instrumentation needed, and lack of sufficiently fast digital electronics. With the present technological developments in digital electronics and easy availability of microwave (MW) components, many laboratories are now exploring the use of Fourier transform techniques. Fully developed pulsed EPR machines are also commercially available from Bruker²⁰. Pulsed EPR makes time-domain EPR measurements and fast data acquisition possible, though it suffers from the limitations caused by the fast EPR relaxation times. The electron spin can be influenced by the nuclear spin, leading to many more interesting pulsed experiments than in NMR

as described in the later sections. Arthur Schweiger made significant contributions in pulsed EPR instrumentation and demonstrated wonderful experiments involving combination of MW, rf and magnetic field pulses to extract a variety of properties²¹.

In 1956, G. Feher proposed electron nuclear double resonance (ENDOR) spectroscopy²² which facilitated the identification of precise details of hyperfine interaction that is observed in EPR spectrum. In the ENDOR technique, the NMR spectrum of a paramagnetic compound is measured indirectly *via* the electron spin. The enhancement of resolution in hyperfine structure occurs from smaller number of ENDOR lines. A spin system having *N* nuclei of nuclear spin $I = \frac{1}{2}$ gives only 2*N* ENDOR lines while an EPR spectrum would have 2^{*N*} lines.

The aim of this paper is to outline the evolution of magnetic resonance from its origin to the current advances. The vast subject of magnetic resonance and its applications would probably take several volumes and hence only a brief but focused presentation would be made here.

2 NMR Spectroscopy

"I am not aware of any other field of science outside of magnetic resonance that offers so much freedom and opportunities for a creative mind to invent and explore new experimental schemes that can be fruitfully applied in a variety of disciplines. NMR spectroscopy is intellectually attractive because the observed phenomena can be understood based on sound theory, and almost all concepts can also be tested by easy experiments. At the same time, the practical importance of NMR is enormous and can justify much of the playful activities of an addicted spectroscopist"

-Richard R. Ernst⁴

"NMR spectroscopy — a technique used by chemists to analyze (materials) molecules. It involves putting the molecules in a magnetic field and hitting them with pulses of radio waves to which they respond with signals that depend on how their spins are aligned... NMR works because in any given bunch of

molecules, there are slightly more spins pointing in one direction rather than another... signal from surplus spins stands far enough out from the background noise for the NMR equipment to pick them up.

The Statesman — Quantum Computers

The transition from a Nobel Laureate's interpretation to that addressed to the common literate man indicates the versatility and use of NMR spectroscopy.

The application of NMR spectroscopy to organic molecules especially the study of chemical shifts of ^1H and ^{13}C nuclei made it an important technique for the characterization of molecular structure. The theory of chemical shifts was extensively developed including effects like influence of neighbouring atoms on chemical shift, ring current effects, hydrogen bonding, solvent effects, molecular dynamics. High resolution NMR (usually measured in CW mode) implemented several experimental methods like improving the homogeneity of external magnetic field by shim coils, sample spinning, spin decoupling by the application of a second rf, measurement at high magnetic fields and signal averaging. The possibility to study the symmetry and chirality of molecules made proton NMR an important investigative technique for biomolecules²³.

2.1 The Nuclear Spin Hamiltonian

Nuclei are well localized within the molecular environment and can be effective reporters on local affairs in their immediate vicinity. Thus, using NMR, the molecular properties can be monitored free from the perturbations caused by the external environment. The complete Hamiltonian of a molecular system is enormously complex in most cases. However, magnetic resonance experiments can be described by a drastically simplified spin-Hamiltonian ($\mathcal{H}$) which is proven to be an asset to this field over many other spectroscopic techniques. The nuclear spin-Hamiltonian contains only the nuclear spin operators and a few phenomenological constants which originate from the reduction process of complete spin-Hamiltonian. The nuclear spin-Hamiltonian can be described by

$$\mathcal{H} = \mathcal{H}_Z + \mathcal{H}_J + \mathcal{H}_D + \mathcal{H}_Q \quad \dots (1)$$

The Zeeman interaction $\mathcal{H}_Z$ with the static field B_0 modified by chemical shielding tensor σ is given by

$$\mathcal{H}_Z = - \sum_{k=1}^N \gamma_k \mathbf{I}_k (1 - \sigma_k) B_0 \quad \dots (2)$$

where the summation is over all nuclei. γ_k is gyromagnetic ratio, $\mathbf{I}_k$ is nuclear spin, and σ_k is shielding tensor of k^{th} nucleus and B_0 is static field. The indirect, electron-mediated interactions which result from electron-nuclear interaction have the form

$$\mathcal{H}_J = 2\pi \sum_{k<l} \mathbf{I}_k \mathbf{J}_{kl} \mathbf{I}_l \quad \dots (3)$$

with indirect spin-spin coupling tensor $\mathbf{J}_{kl}$ for the spins $\mathbf{I}_k$ and $\mathbf{I}_l$. The anisotropic part of $\mathbf{J}_{kl}$ can be separated as

$$\mathbf{J}_{kl} = \mathbf{J}_{kl}^{\text{iso}} + \mathbf{J}_{kl}^{\text{aniso}} = J_{kl} + \mathbf{J}_{kl}^{\text{aniso}} \quad \dots (4)$$

where $\text{tr}\{\mathbf{J}_{kl}^{\text{aniso}}\} = 0$, and $J_{kl} = \frac{1}{3} \text{tr}\{\mathbf{J}_{kl}\}$, leading to

$$\mathcal{H}_J^{\text{iso}} = 2\pi \sum_{k<l} J_{kl} \mathbf{I}_k \mathbf{I}_l \quad \dots (5)$$

$$\mathcal{H}_J^{\text{aniso}} = 2\pi \sum_{k<l} \mathbf{I}_k \mathbf{J}_{kl}^{\text{aniso}} \mathbf{I}_l \quad \dots (6)$$

In the context of high resolution NMR in liquid phase, we shall often encounter the spin-Hamiltonian

$$\mathcal{H} = - \sum_{k=1}^N \gamma_k (1 - \sigma_k^{\text{iso}}) B_0 I_{kz} + 2\pi \sum_{k<l} J_{kl} \mathbf{I}_k \mathbf{I}_l \quad \dots (7)$$

Direct dipolar interactions between the nuclear magnetic moments provide structural information. The dipolar Hamiltonian has the form

$$\mathcal{H}_D = \sum_{k<l} \mathbf{I}_k \mathbf{D}_{kl} \mathbf{I}_l \quad \dots (8)$$

or explicitly,

$$\mathcal{H}_D = \sum_{k<l} b_{kl} \left\{ \mathbf{I}_k \mathbf{I}_l - 3 \frac{1}{r_{kl}^3} (\mathbf{I}_k \mathbf{r}_{kl}) (\mathbf{r}_{kl} \mathbf{I}_l) \right\} \quad \dots (9)$$

where $b_{kl} = \mu_0 \gamma_k \gamma_l \hbar / (4\pi r_{kl}^3)$ in SI units and $\mathbf{r}_{kl}$ is the internuclear distance vector.

The electric quadrupole interaction may be

interpreted as nuclear interactions with electric field gradients. These are present only for the nuclei having spin $I > \frac{1}{2}$. The quadrupolar Hamiltonian term is given by

$$\mathcal{H}_Q = \sum_k \mathbf{I}_k \cdot \mathbf{Q}_k \cdot \mathbf{I}_k \quad \dots (10)$$

where $\mathbf{Q}_k$ is the quadrupole coupling tensor which can be expressed in terms of electric field gradient tensor $\mathbf{V}_k$ at the site of nucleus k

$$\mathbf{Q}_k = \frac{eQ_k}{2I_k(2I_k + 1)\hbar} \mathbf{V}_k \quad \dots (11)$$

where Q_k is the nuclear quadrupole moment of nucleus k .

2.2 Evolution of FT NMR

The low sensitivity of nuclear spin interaction lead to severe problems in cw detection. A large number of spins is required under optimum conditions, e.g. with a modern high field NMR spectrometer 10^{14} to 10^{15} spins of one kind are required to detect a signal within a measurement time of 1 hr. A few approaches considered for optimizing sensitivity are:

- a) Measurement at high field
- b) Use large bulk of sample
- c) Cooling the sample to increase Curie magnetization
- d) Enhancement of magnetization by special techniques like heteronuclear Overhauser effect, and electron nuclear Overhauser effect
- e) Indirect detection of resonance by (i) Electron nuclear double resonance (ENDOR), (ii) Internuclear double resonance (INDOR), (iii) Cross-polarization in solids and liquids, (iv) Transfer of coherence by rf pulses
- f) Flowing sample techniques to circumvent saturation effects
- g) Shortening T_1 by addition of paramagnetic materials
- h) Signal averaging

Initial efforts were on building a multichannel spectrometer, which would allow simultaneous measurement of numerous points thereby reducing spectral acquisition time. Such an attempt that was undertaken by Weston A Anderson at Varian Associates²⁴ soon revealed that the instrumental effort becomes exorbitant when the number of

channels was increased. The search for a solution to this problem lead to the use of pulsed FT NMR spectroscopy^{5,25}. In this method, a strong rf pulse is considered as a multifrequency rf exciting source and the FT of impulse response returns a frequency spectrum. The construction of FT NMR spectrometer gave the advantage of higher sensitivity, resolution and the absence of lineshape distortions. Now-a-days most laboratories have replaced the CW NMR machines with pulsed instruments to exploit these advantages.

A schematic diagram of 1-dimensional FT NMR technique is shown in Fig. 1. The sample is polarized in a static magnetic field along the z-axis. Application of a $\pi/2$ rf pulse along y-axis rotates the magnetization vectors of spins by 90° to an orientation perpendicular to static field. The relaxation processes would cause free induction decay (FID) of this impulse response which consists of a superposition of all eigenmodes. It is Fourier transformed from time-domain to frequency-domain using a digital computer and the signal is stored in its memory. It is possible to consider other methods for a multifrequency source instead of a delta-function like rf pulse e.g. stochastic random noise excitation, rapid scan excitation, excitation by computer simulated multiple frequency waveform. These involve different methodology and instrumentation for the detection of the spectrum⁴.

2.3 2D-FT NMR Spectroscopy

The FT NMR technique described above gives chemical shifts along frequency axis $S(\omega)$ in one dimension. This is adequate for the characterization of chemical environment of nucleus. However, it does not yield any spatial relationships between the nuclei. The through bond spin-spin coupling J and through space spin-spin coupling D can be studied using double- or triple- resonance experiments in which two or three rf fields are applied simultaneously. In principle, it is possible to map the amplitude $S(\omega_1, \omega_2)$ into two dimensions by these methods. These multiple resonance experiments can be replaced by multiple pulse experiments in FT spectroscopy as discussed by Jean Jeener²⁶ and Richard Ernst⁶.

Fig. 2 shows the pulse sequence for a 2D-FT experiment. A $\pi/2$ preparation pulse excites the coherences (transitions) for an evolution time t_1 .

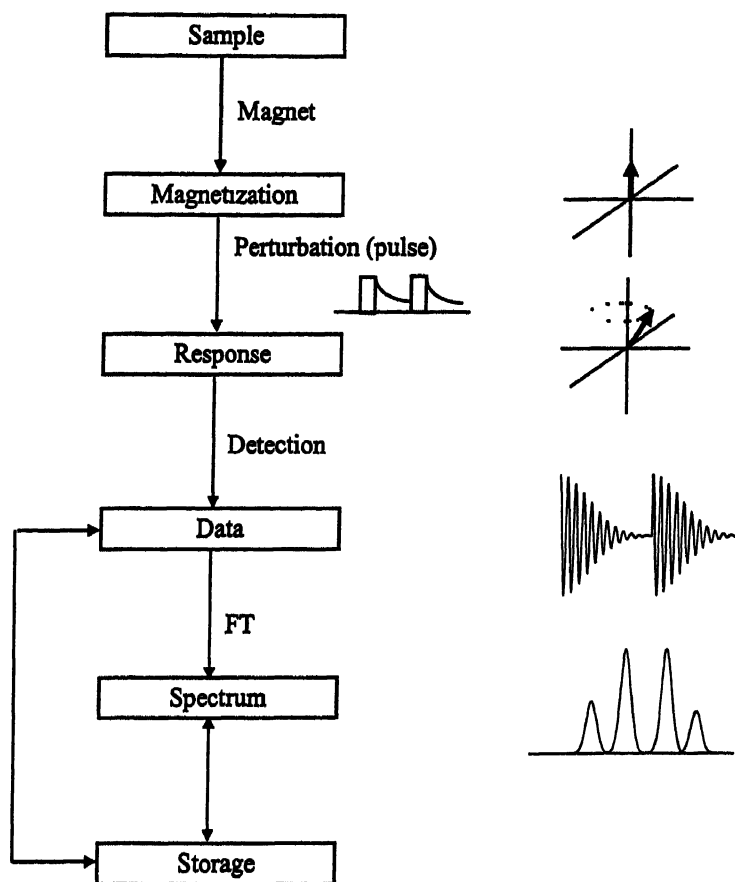


Fig 1 Schematic diagram of a Fourier Transform NMR spectral acquisition

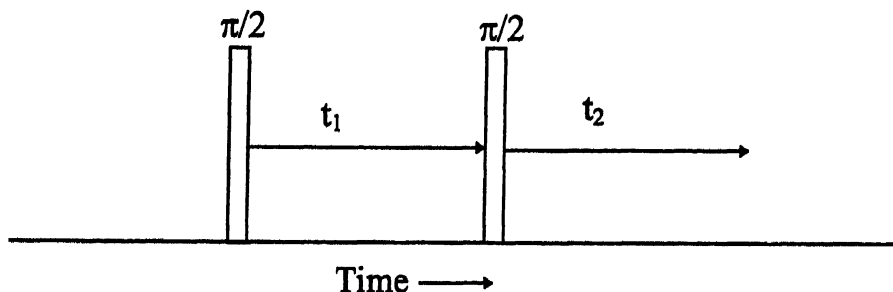


Fig. 2 Pulse sequence for a two-dimensional Fourier transform experiment. The first pulse excites coherences that precess during t_1 and are transferred by the second pulse to different transitions where the coherences continue to precess with a new frequency

During this period, the coherences are frequency labeled. Subsequently a $\pi/2$ mixing pulse is applied which performs a controlled transfer of coherence to different nuclear spin transitions that evolve during detection period t_2 . To obtain a 2D-spectrum $S(\omega_1, \omega_2)$, it is necessary to carry out a series of two-pulse experiments with systematically varied t_1 values, then assemble the 2D-data matrix, and Fourier transform it in two dimensions⁴. This

2D-spectrum correlates the precession frequencies during evolution with the frequencies during detection. The scalar J couplings can be correlated this way which is called 2D-correlation spectroscopy (COSY).

Similar pulse sequences can be applied to map a variety of properties as shown in Fig. 3. Internuclear cross relaxation leads to 2D-nuclear Overhauser effect spectroscopy (NOESY).

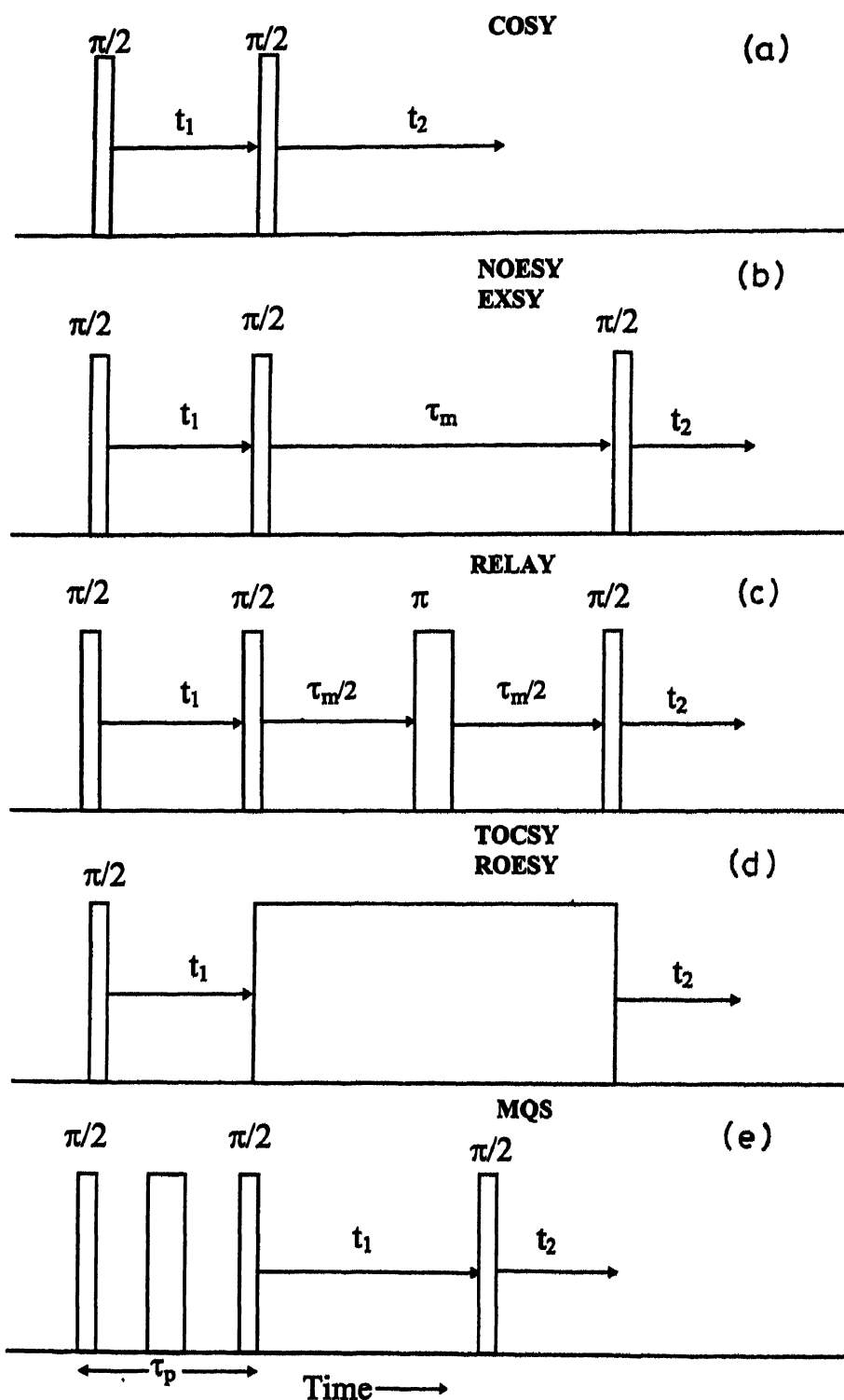


Fig. 3 Pulse sequences for homonuclear 2D experiments: (a) COSY, (b) NOESY or EXSY (c) relayed COSY, (d) TOCSY or ROESY in the rotating frame (e) Multiple-quantum spectroscopy

Chemical exchange leads to 2D-exchange spectroscopy (EXSY). These involve incoherent, dissipative processes that bring the system back to equilibrium in an exponential or multiexponential

manner after the first pulse. The random processes occur during this time. Both processes can be investigated with the same three-pulse sequence as shown in Fig. 3. The mixing period is bracketed by

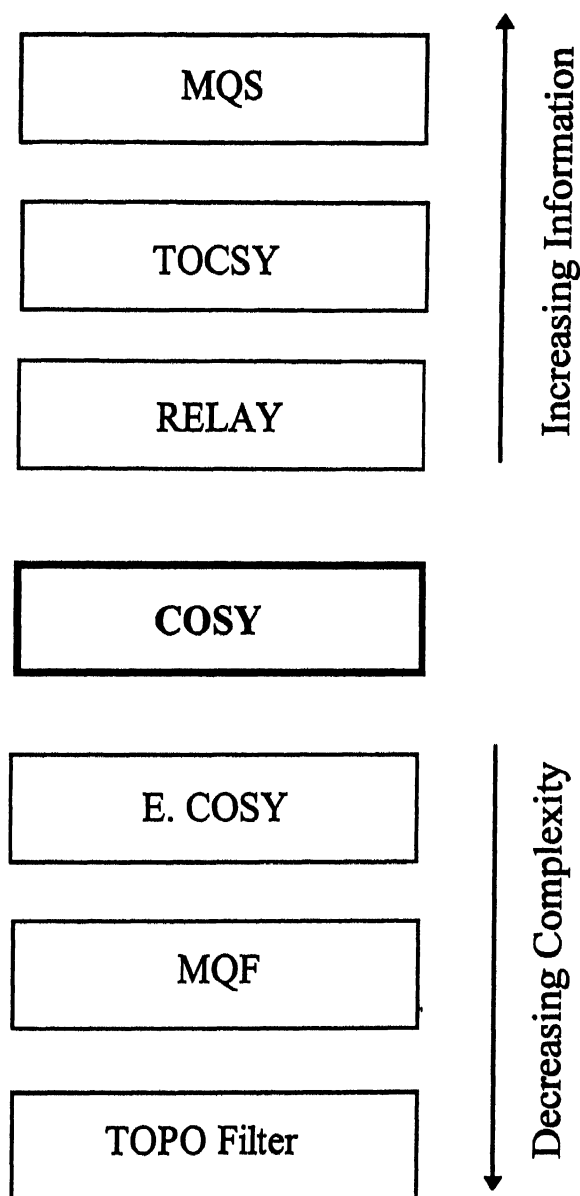


Fig 4 Extensions of the COSY experiments. Relayed correlation, TOCSY, and MQS increase the information content. E COSY, MQF and spin-topology filtrations reduce the complexity. Both avenues can lead to 3D spectroscopy

two $\pi/2$ pulses that transform coherence into static spin-order and back into coherence. For NOESY transfer, the exchange rate constants are given by the cross-relaxation rate constants, which give distance dependencies.

Numerous modified, expanded and improved 2D-FT NMR experiments have been suggested in literature, which are schematically shown in Fig. 4. One category of experiments, represented in the upper part of the Fig. 4 provides more detailed information. These are relayed correlation

experiments (involving two-step correlation), total correlation spectroscopy (TOCSY) and Multiple-quantum spectroscopy (MQS). TOCSY leads the important class of rotating frame experiments, including rotating frame Overhauser effect spectroscopy (ROESY) which is an alternative to NOESY. MQS allows one to investigate connectivity in spin systems. The other category of experiments attempt at the simplification of spectra by exclusive correlation (E. COSY), multiple-quantum filtering (MQF), and spin-topology filtration.

3D-NMR Spectroscopy is a natural extension of 2D-NMR spectroscopy. Instead of a single mixing process which relates two frequency variables, two sequential mixing processes relate three frequencies: the original (first evolution) frequency ω_1 , the second evolution frequency ω_2 , and the detection frequency ω_3 . Though a large number of possible 3D-experiments can be conceived, only a few of them have proved to be indispensable so far.

The molecular structures determined by NMR are essentially motionally averaged. Hence it is important to note the motional properties of the molecule. The correlation times τ_c that can be covered by NMR range from picoseconds to greater than a second. While real time monitoring after the pulse is possible for $\tau_c > 1$ s, T_1 measurements can be done for cases where τ_c is between 30 ps and 1 μ s. The 2D-EXSY methods measure τ_c values between 10 ms – 1 s.

2.4 Magnetic Resonance Imaging (MRI)

The basic procedure for recording a 2D- or 3D-NMR image of an object is attributed to Paul Lauterbur⁷. The NMR spectrum measured in a magnetic field gradient—instead of a homogeneous magnetic field—contains both spectral and spatial information. Deconvolution of spectral part from this yields a projection of spin density at a view angle that is determined by the gradient direction. Application of gradients along different directions in space in a sequence of experiments produces a set of projections. The image is reconstructed from these projections using filtered backprojection technique or by any other method in analogy to X-ray tomography.

A different approach directly related to this can be used in 2D- and 3D-FT spectroscopy as described by Ernst⁴. Phase and frequency encoding

of the three spatial dimensions is achieved by a linear magnetic field gradient applied successively along three orthogonal directions for the durations t_1 , t_2 , and t_3 respectively in a pulsed FT experiment. The recorded signal $s(t_1, t_2, t_3)$ is Fourier transformed in three dimensions to produce a function $S(\omega_1, \omega_2, \omega_3)$ which is equivalent to a 3D-spatial image when the spatial information is decoded using the relations $x = \omega_1/G_x$, $y = \omega_2/G_y$ and $z = \omega_3/G_z$ with the three field gradients G_x , G_y and G_z .

3 EPR Spectroscopy

"... So much so (three decades after the discovery of EPR) that a decline appeared to set in and many chemistry departments, regarding EPR primarily as an analytical tool, shifted their research effort in spectroscopy to other techniques. . . About 10 years ago pulsed EPR techniques, albeit devised two decades earlier, began to take off with the general availability of low-cost microwave switches and solid-state amplifiers and with the development of special cavities. The penetration of EPR into biological research and the development of new techniques have led to, and are the result of, founding of several EPR centers devoted to the biological applications of EPR and ENDOR. This in turn has led to a rather spectacular growth in the number of applications in which advanced EPR techniques are used to tackle problems of biological interest."

-A J Hoff²⁷

The rapid development of EPR spectroscopy at its initial stages may be attributed to its variety of applications and extraction of detailed information on paramagnetic centers and free radicals. Many interesting experiments were carried out discovering exciting results and theories were developed for the interpretation in the study of transition metal complexes²⁸, extended interactions of magnetically coupled molecules²⁹, dynamics of long chain biomolecules associated with transition metal ions, spin-labeling the biomolecules²⁹, free radicals *in vivo*, spin-trapping²⁷, radiation damage³⁰ ferro and antiferromagnetic resonance, superconductivity³¹ and so on.

An apparent difference between EPR and NMR is that EPR covers a larger frequency range (*viz* wide range of g -values) than NMR, because the electron spin is easily influenced by other electrons and nuclei in the vicinity. Conventionally NMR spectrum is presented in frequency scale while EPR used magnetic field scale. Large number of EPR measurements reported in literature were carried out at X-band frequency (9.5 GHz) for its sensitivity, convenient cavity dimensions and ease of instrumentation. However, the spectrometers operating at other frequencies L-band (1-2 GHz), S-band (6 GHz), K-band (17 GHz), Q-band (35 GHz), (the naming adapted from their original notation used in defense research) and recently as high as 370 GHz³² and as low as 200 MHz are also known.

3.1 The EPR Spin-Hamiltonian

The EPR spin-Hamiltonian can be described as

$$\mathcal{H} = \mathcal{H}_{EZ} + \mathcal{H}'_{SI} + \mathcal{H}'_{SS} + \mathcal{H}'_Q + \mathcal{H}'_{II} + \mathcal{H}_{NZ} + \mathcal{H}_{exch} \quad \dots (12)$$

$\mathcal{H}_{EZ}$ is the interaction of the electron spin S with external field B_0 expressed by

$$\mathcal{H}_{EZ} = \beta_e S \cdot g \cdot B_0 \quad \dots (13)$$

As the Zeeman energy can also be affected by orbital angular momentum and crystal field effects, the g -tensor yields information about excited electronic states and symmetry of the environment of a paramagnetic center. The term $\mathcal{H}'_{SI}$ describes the hyperfine interactions between the electron spin and the nuclear spin, I .

$$\mathcal{H}_{SI} = h S \cdot A \cdot I \quad \dots (14)$$

where A is hyperfine coupling tensor. The $\mathcal{H}'_{SS}$ term yields the information about spin-spin interaction.

$$\mathcal{H}_{SS} = S \cdot D \cdot S = g^2 \beta_e^2 \left[\frac{(S_k \cdot S_l)}{r^3} - \frac{3(S_k \cdot r)(S_l \cdot r)}{r^5} \right] \quad \dots (15)$$

where r is the inter-electronic distance vector and D is dipolar tensor. The terms $\mathcal{H}'_Q$, $\mathcal{H}_{II}$ and $\mathcal{H}_{NZ}$ describe the nuclear quadrupolar energy, nuclear spin-spin coupling and nuclear Zeeman

energy. $\mathcal{H}_\Pi$ and $\mathcal{H}_{NZ}$ interactions are weak and generally less important in EPR. The $\mathcal{H}_{\text{exch}}$ term represents the exchange interaction between two paramagnetic centers k and l , coupled by and exchange coupling constant J_{kl} where

$$\mathcal{H}_{\text{exch}} = J_{kl} S_k \cdot S_l \quad \dots (16)$$

3.2 Resonators of EPR Spectrometer

An X-band EPR spectrometer basically contains a klystron or Gunn diode microwave source, a circulator, a resonant cavity, a detector crystal, detection system and a magnet. The detection system conventionally employed a 100 kHz AC modulation signal and phase sensitive detection in order to enhance the signal to noise ratio which results in a first derivative plot of microwave absorption.

The resonant EPR cavities are specially designed with specific dimensions and geometry. e.g. the rectangular TE_{102} cavity used at X-band frequency has dimensions of one and two half wavelengths of microwave in the plane perpendicular to applied field. The cylindrical TE_{011} cavity holding very high-energy density has one half wavelength height and diameter. These cavities have high quality factor Q , (typically an unloaded Q of 5000) where

$$Q = 2\pi (\text{max. MW energy stored}) / \text{energy dissipated per cycle} \quad \dots (17)$$

The scene in EPR cavity design has changed with the advent of lumped circuit resonators especially of the style of loop-gap resonators

(LGR) described by Hyde and Froncisz³³. LGRs are flexible structures, which allow one to design the resonator to fit the experiment rather than to adapt the experiment to the fixed size and shape of cavity. The LGR can be made smaller than cavity resonators permitting their use in lower frequency EPR spectrometers. A variety of loop gap resonators—slotted tube, bridged loop-gap, copper tape, folded halfwave, reentrant and copper film, have been reported in literature. An LGR basically contains a conductive cylindrical loop in which one or more capacitive gaps are created and an inductive coupler. Schematic diagram of an LGR with one loop and two gaps described by Jim Hyde is shown in Fig. 5. The LGR has very low Q value typically about 600 at X-band frequency making them ideal for pulsed measurements³⁴. In pulsed experiments, the ring down time of resonator after the pulse, which is called dead time inhibits the detection of FID. The low Q of LGR reduces this dead time.

3.3 Former Trends in EPR

A large extent of EPR spectroscopic studies initially dealt with the quantitation of spin-Hamiltonian parameters of transition metal ions and ligand hyperfine coupling constants. A comprehensive compilation was presented by Kuska and Rogers for various transition metal complexes³⁵. The spin-Hamiltonian parameters and molecular orbital coefficients, spin densities and spin-orbit coupling values were determined for

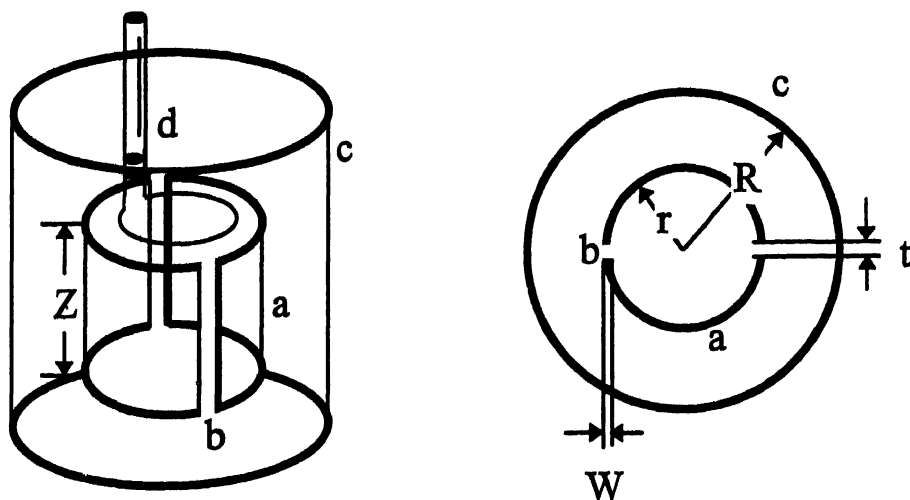


Fig. 5 Schematic diagram of a loop-gap resonator showing the principal components: (a) loop, (b) gaps; (c) shield; (d) inductive coupler

various oxidation states of metal ions. The experimental methods in EPR spectroscopy and the theory for the extraction of these parameters is discussed in detail in several text books²⁸. These include measurement techniques of EPR spectrum in low viscous solutions, single crystals, polycrystalline medium, and temperature dependent measurements, frequency dependent measurements, magnetic susceptibility studies, extraction of zero-field splitting terms and quadrupolar couplings.

EPR line shape studies and exchange coupled systems were extensively reported in literature. Through bond and/or through space electron exchange over an infinite chain of molecules in a crystalline lattice modulates the magnetic properties of individual molecules in the chain. A strong exchange results in a line narrowing effect and the quantitation of exchange coupling is carried out by line shape studies³⁶. A very weak exchange coupling of electron can lead to an entirely different EPR spectrum making EPR a sensitive sensor to detect very small energy changes. EPR study of a series of weakly coupled liquid crystalline compounds was reported recently³⁷. The electron spin-exchange in single crystal lattice causes an increase in conductivity along the exchange direction. Conductivity studies and magnetic susceptibility studies as a function of temperature may also be used to determine the strength of exchange coupling.

Biological applications of EPR were predominantly carried out on spin-labelled biomolecules. Spin-labels generally contain nitroxide free radical with characteristic intense narrow EPR spectral lines. These molecules are attached to the biomolecules such as the enzymes (lysozyme, ribonuclease, DNA Polymerase, citrate synthase), lipids, peptides and aminoacids to study their activity in the system³⁸. The conformational changes of biomolecules are studied using biradicals. Biradicals are molecules containing two radicals in a single molecule connected by a long chain. The two radicals exchange their spins leading to spin-exchange. In solution, the molecules are present in several conformations leading to dynamic changes in the exchange coupling between the radicals. This results in a complex time dependent spectrum³⁹. Numerous EPR studies involving biomolecules like hemins, chlorophyllins, polypeptides, amino acids were

studied by substitution or introduction of different transition metal ions⁴⁰. In these studies the EPR spectrum of transition ion reveals the ligand surroundings and their dynamics leading to an understanding of the molecule under investigation.

It has been well established that many biological reactions are accompanied by free radical mechanisms. The enzymatic catalysis is believed to be through free radical intermediates. The study of superoxide and hydroxyl radicals are of great importance in cancer research. Nitric oxide is believed to be an important relaxant of the blood vessels. Study of these transient free radicals that are highly reactive and destroyed at diffusion controlled rates after their production has been a challenge to chemists. Spin-trapping methods are developed for the study of these transient radicals. A spin-trap is an EPR insensitive molecule by itself but forms an adduct by extracting a free radical thereby becoming EPR active. The spin-trapping techniques were reported by various authors in literature⁴¹ though it is still a challenge to precisely determine the free radicals *in vivo*.

3.4 Current Themes in EPR

The availability of microwave components, digital electronics at low prices, and the invention of LGR are setting grounds for new trends in EPR. EPR is now becoming multifrequency, multidimensional and time domain. The resonators are being built for specific purposes and the gradient fields are used as desired for novel experiments. Biological applications of EPR are increasing including the studies of free radicals like superoxide, hydroxyl radical, nitric oxide and oxygen *in vivo* using both CW and FT EPR techniques. The current themes of EPR are listed in Table I. Many of these involve FT EPR, low frequency EPR and custom designed resonators for specific applications.

3.5 FT EPR Spectroscopy

In principle, Fourier transform EPR spectroscopy is similar to FT NMR except for the use of MW pulse instead of rf pulse. In FT NMR, it is usually possible to achieve complete and simultaneous excitation of the entire spectrum using a single short rf pulse. However, in the corresponding EPR experiment, this is only possible for limited compounds. Problems arise

Table I
Recent topics in EPR spectroscopy

Multi-dimensional FT EPR COSY, EXSY, HSCORE, HYEND
ESEEM studies on Metalloproteins, disordered systems
CW and Pulsed ENDOR studies of metalloenzymes, heme-globin interactions
Time Resolved EPR.
Multi-frequency ESEEM studies
Magnetic field dependence in ESEEM studies
Optically detected magnetic resonance of triplet states
Multipulse techniques.
New EPR pulse sequences
Applications of ESEEM to metalloenzymes.
Multiple frequency electron spin echo.
Emphasis on the time domain as well as on the frequency domain.
Interpreting electron spin echo data
Saturation-recovery EPR.
Very high frequency EPR.
Non-linear CW EPR
EPR without magnetic field modulation
Multiple quantum EPR
Fast-response EPR
ENDOR of metal ions
Spin label studies for dynamics of lipids and proteins.
<i>In vivo</i> EPR.
Very low frequency <i>in vivo</i> EPR to develop diagnostic methods in medicine.
Oximetry combined with imaging used in medical diagnosis.
Spin-trapping studies <i>in vivo</i> .
EPR imaging for the <i>in vivo</i> study of free radicals in biology.
Design and construction of LGRs.
Resonators designed to fit the needs of the experiment.
Practical aspects of spectrometer construction
Slow-wave and non-resonant microwave structures
Calculational and experimental aspects of molecular motion.
Mathematical methods for the interpretation of time-domain EPR.
Software standards and portability in the EPR community
EPR simulation problems.

with the generation of strong MW pulse, the limited bandwidth of detection system and necessity for very rapid data acquisition. Often only a portion of the spectrum is excited and large distortions in the intensity and phase occur. The distortions can be minimized by using quadrature detection²¹. Another problem is the dead time of the pulsed EPR spectrometer that prevents observation of FID during the first 50-100 ns after the pulse. It is therefore difficult to measure broad resonance lines using FT EPR in the near future.

In principle, FT EPR method makes it possible to record entire EPR spectrum within a few

microseconds. This allows detection of transient species²¹. A variety of pulsed EPR techniques are reported in literature. Similar to 2D-NMR, two-dimensional FT EPR methods were also developed though these are subjected to the limitations in 1D-FT EPR mentioned above. The pulse sequences and methodology for 2D-correlation spectroscopy (COSY), 2D spin echo correlation spectroscopy (SECSY), 2D Exchange spectroscopy (EXSY) and 2D ESEEM techniques were described by A Schweiger²¹. The electron spin-echo techniques (ESE) provide an easy way to separate static inhomogeneous line broadening from homogeneous broadening caused by dynamic effects. Electron spin-echo envelope modulation (ESEEM) studies are useful for the determination of anisotropic interactions present in solids.

3.6 Electron Spin Echoes

The spin echo was first described by Hahn in the case of nuclear spin and by Blume⁴² in the case of electron spin. The rapid decay of the FID caused by inhomogeneous broadening can be reversed by issuing a 180° refocusing pulse after time τ .

A schematic diagram of an echo experiment is shown in Fig. 6(a). The echo phenomenon depends on the non-linear behaviour of an ensemble of oscillators with different frequencies. A two pulse echo experiment involves a $\pi/2$ exciting pulse and a π refocusing pulse separated by a time interval τ . The echo is observed at a time τ after the refocusing π pulse. The amplitude of two pulse echo is a function of pulse interval τ and its rate of decay which is described by the phase memory time T_M . The phase memory time is defined as the time taken for the echo amplitude to decay to $1/e$ of its initial value.

Fig. 6(b) shows the pulse sequence to generate a stimulated echo. This is a three $\pi/2$ -pulse sequence with pulse intervals τ and T . The z -magnetization is transferred to transverse magnetization by first $\pi/2$ pulse. It is dephased in xy -plane for a time τ . The result of second $\pi/2$ pulse is rotation of magnetization into xz -plane. A third $\pi/2$ pulse applied after time $T \leq T_1$, generates an echo of this magnetization pattern. It consists of a single peak at a time τ after the pulse and is called a stimulated echo. The three-pulse sequence also generates several two-pulse echoes (normal spin-echoes).

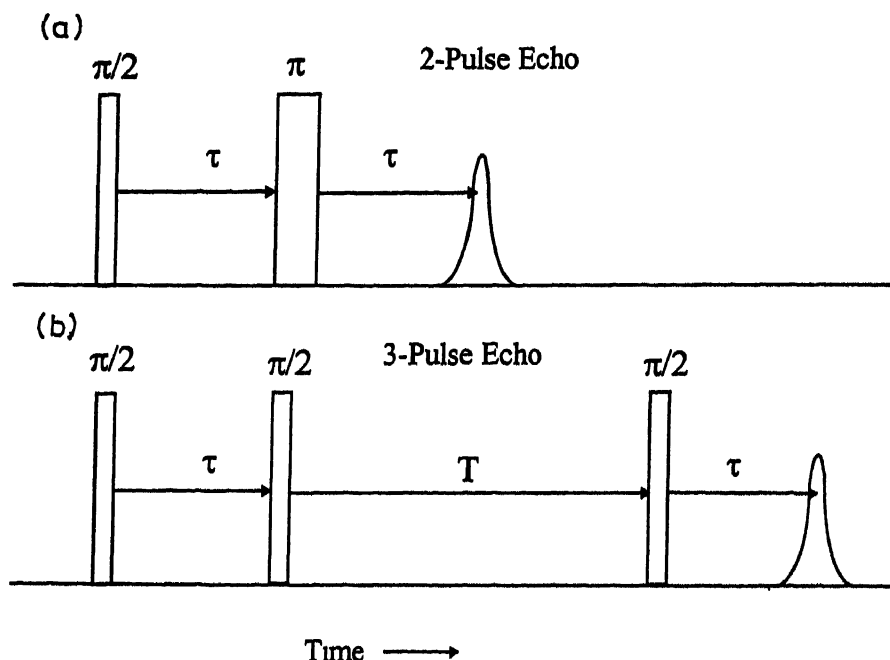


Fig 6 (a) Two-pulse echo sequence (b) Three-pulse echo sequence

These can be eliminated by means of a phase cycle. Phase cycles in pulsed EPR provide a way of suppressing unwanted contributions to the time-domain signal. Since EPR spectra are often poorly resolved the FID and therefore also the spin echo, show little structure in these cases. For this reason the amplitude of the echo is used as the main source of information in spin-echo experiments. Measuring the intensity of two-pulse or three-pulse echo as a function of applied field can be considered as an alternative to CW EPR. The advantage of pulsed techniques is the time resolution at microsecond scale. FT EPR does not require field modulation while the line shape and sensitivity of a CW EPR spectrum depend on modulation amplitude. Thus FTEPR is useful for the measurement of kinetics of short lived free radicals and also possibly for the study of transient phenomena like chemically induced dynamic electron spin polarization (CIDEP).

The relaxation times can be determined using spin echo methods. In echo methods, all types of interactions that can cause inhomogeneous broadening undergo refocusing. This makes it easy to separate inhomogeneous contributions to the linewidth from homogeneous broadening caused by dynamic effects. The spin-lattice relaxation time T_1 can be determined from the amplitude of stimulated echo, as the z -magnetization relaxes

towards thermal equilibrium with time. To avoid spectral diffusion problems in this technique, one uses saturation recovery method where a long pulse or succession of many short $\pi/2$ pulses are used to saturate EPR signal. The measurement of z -magnetization after time T can be done either by recording FID or by using a two-pulse echo.

3.7 Electron Spin Echo Envelop Modulation (ESEEM)—The Nuclear Modulation Effect

In the two or three pulse ESE experiments, the couplings between electron spin and nuclear spins can cause modulation of echo intensity. This is called electron spin echo envelope modulation (ESEEM). This can only occur if there are anisotropic interactions present in the system⁴¹. In a system containing e.g. $S=1/2$ and $I=1/2$, there are two allowed and two forbidden transitions. During the evolution period in ESE experiments, the magnetization vectors corresponding to the allowed transitions precess with MW frequency (ω_{MW}) while the forbidden transitions precess with difference frequencies ($\omega_{\text{allowed}} - \omega_{\text{forbidden}}$). At the time of refocussing, the spin echo of an allowed transition is modulated by the forbidden transition, since the allowed and forbidden transitions involve a common energy level. Further, it leads to the appearance of extra lines at combination and

difference frequencies between allowed and forbidden transitions.

The two pulse ESEEM is somewhat similar to ENDOR spectrum (which will be discussed later) except for extra lines due to combination frequencies. The nuclear modulation effect arises solely from the interplay of allowed and forbidden transitions without the need for an external rf field. In a two-pulse echo modulation, the dead time can cause serious problems when the phase memory time T_M is small. A three pulse ESEEM decays much slower than a two pulse ESEEM and has smaller number of combination lines compared to two pulse ESEEM. This is especially suitable for single crystal studies. A number of pulsed EPR detection schemes, which were developed recently are given in Table II.

3.8 2D-FT EPR Spectroscopy

Two-dimensional FT EPR spectroscopy follows

Table II
New EPR detection schemes

Two and three pulse electron spin echoes
Two and three pulse ESEEM
4-Pulse ESEEM
5-Pulse ESEEM
Soft ESEEM
2+1 pulse train ESE
Remote echo detection
Echo modulation echoes
ESEEM at frequencies other than X-band
Radio-frequency driven ESEEM
FID detected hole burning
Longitudinal detection
New methods for the measurement of the nuclear modulation effect
Phase shifted excitation
Extended time excitation
Coherent Raman beats
2D FT-EPR in solids
Phase cycling
Double resonance experiments
Triple resonance
Fourier transform EPR-detected NMR
Optimized ENDOR
HYSCORE - Hyperfine selected ENDOR
Multiple quantum ENDOR
Time-domain ENDOR
Coherence transfer ENDOR
SEDOR-ENDOR Spectroscopy
1D and 2D pulsed ELDOR
Spectral-spatial EPR imaging
Resolution enhancement of field swept EPR
Electron-Zeeman resolved EPR
Anisotropy-resolved EPR
Electron spin transient nutations.

similar methods that are applied in NMR, however, with the limitations that apply to 1D-FT EPR. The COSY experiment can be used to study the coherence transfer between the coupled electron spins. 2D-Exchange spectroscopy (EXSY) is useful for monitoring the transfer of magnetization between hyperfine lines.

The ESEEM technique can also be used for 2D experiments as in the case of COSY. For the study of structural information, a four pulse ESEEM as shown in Fig. 7 is more useful than a two pulse ESEEM. In two-pulse ESEEM, one would observe extra lines due to sum and difference frequencies in addition to the modulation effect. The positions of these lines contain information about dipolar part of hyperfine couplings. The phase memory time T_M is short for a two pulse ESEEM. A four pulse ESEEM takes the advantage of slow decay of echo intensity as in the case of three-pulse ESEEM and the appearance of sum frequencies as in two-pulse ESEEM. A 2D four pulse ESEEM gives a 2D map of hyperfine couplings and their signs. This is also called HYSCORE (hyperfine sublevel correlation) experiment⁴⁴.

3.9 Electron-Zeeman-Resolved EPR

It is common practice in EPR spectroscopy to employ higher frequencies (e.g. Q-band) for the measurement of spectrum when the spectral lines which are g value dependent (e.g. g_x , g_y , g_z) are unresolved at lower frequencies (e.g. X-band). Electron-Zeeman resolved EPR (EZREPR) allows one to expand the field-swept EPR spectrum in a second dimension, representing the electron Zeeman frequency thus providing higher resolution on g value dependent spectral lines.

For a paramagnetic center with $S=1/2$ and nuclear spin I , at the resonance frequency ω_{mw} , there would be $2I+1$ lines with the field positions

$$B_0 = \frac{\hbar}{g\beta_e} (\omega_{mw} - m_I a) \quad \dots (18)$$

If there are several paramagnetic centers with overlapping lines of differing g values, EZREPR technique allows them to be separated by mapping the hyperfine structure in the second dimension at each g value. Sierra, Schweiger and Ernst suggested two pulse schemes for this purpose. One scheme uses a primary ESE sequence, and the other uses a hole-burning experiment followed by detection of FID⁴⁵. In primary ESE technique, a

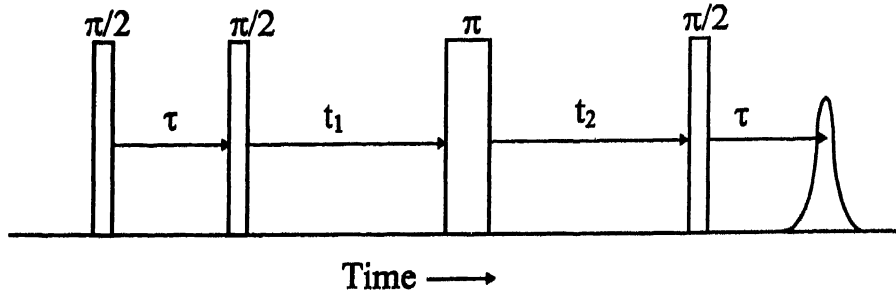


Fig. 7 Pulse sequence of Four-pulse ESEEM

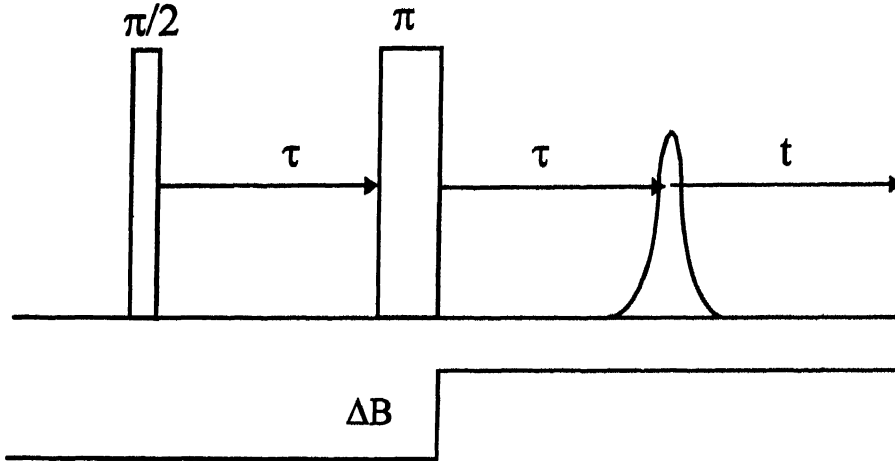


Fig. 8 Pulse sequence used for electron-Zeeman-resolved EPR

conventional two-pulse echo sequence is used as shown in Fig. 8. However, a magnetic field ΔB is applied after the refocusing π pulse. This leads to a modulation of the echo signal $I_x^{echo}(\tau, t)$ as a function of τ and t with the frequency

$$\Omega = \frac{g\beta_e}{\hbar} \Delta B. \quad \dots (15)$$

Fourier transform of the echo amplitude for $t = 0$ as a function of τ yields g -value spectrum with the peak frequencies of $\Omega_k = \frac{g_k\beta_e}{\hbar} \Delta B$. The experiment is repeated for a set of B_0 values resulting in a 2D-EPR spectrum $S(B_0, \Omega)$.

3.10 EPR Imaging

The success of NMR imaging prompted the development of EPR imaging (EPRI) using both CW and pulse techniques following the principles of NMR imaging. The interest in EPR imaging is focused on spectral-spatial imaging which obtains the images of the spectral properties in addition to the spin-density map providing information on

oxygen concentration, free radicals present in the organs of living animals. Unlike magnetic resonance imaging (MRI) which images the huge bulk of protons available in human organs, EPRI has to deal with the trace amounts of paramagnetic species present in the body. Thus usually, EPRI *in vivo* requires injection of spin probes having long relaxation times into the body of the subject. In order to have its application in biology, a real time imaging is desired for which pulsed EPR imaging at radio frequencies is suitable. Several research groups are progressively working on the development of EPRI methods suitable for medical diagnosis.

The spectral-spatial EPRI at X-band frequency using CW methods were described by Maltempo, and Eatons in 1980s⁴⁶. A concise description of experimental aspects for EPRI on a CW X-band machine and the mathematical aspects are briefed by Sotgiu *et al.*⁴⁷ The magnetic field z -gradient is usually generated using a pair of anti Helmholtz coils while the x or y gradient is generated by four D shaped coils. A series of spectra are measured at $G_z = dB_z/dz$, $G_x = dB_z/dx$, $G_y = dB_z/dy$ gradients

which can be related to gradient direction. Each spectrum corresponds to a projection. A filtered back projection method is applied to reconstruct the spectral-spatial image.

The initial EPR imaging experiments carried out by CW machines restricted the imaging to three dimensions (either one spectral and two spatial dimensions or all three spatial dimensions) because of time factor. In the mean time, FT EPR imaging spectrometers have been developed⁴⁸ operating at low frequencies suitable to animal studies. Though, in principle, pulsed EPR spectrum can be acquired in a microsecond, FT EPR imaging requires fast switching gradients. The gradient rise and fall times are generally high and these govern the speed of data acquisition.

3.11 *In vivo* EPR

It is challenging to measure the EPR spectrum in living animals because of restricted access and dimensions of sample volume in the instrumental setup and still emerging developments in methodology. The low frequency EPR machines containing novel LGRs and surface coils combined with FT techniques are meeting these needs. Several narrow line spin probes capable of targeting the organs of living animals are being developed. EPR imaging on animals is carried out by several researchers to study the action of brain, heart, liver and tumor growth. The attempts of *in vivo* EPR imaging of drug distribution and oxygen and nitric oxide mapping are promising. *In vivo* spectroscopy is also focussed on spin-trapping techniques to determine the activity of transient species including nitric oxide, superoxide, hydroxy radical etc., spin-labelling studies to measure oxygen, viscosity fluids in different organs and tumors.

At X-band frequency, the microwave penetrates only a millimeter into biological specimens because of dielectric loss. Calculations indicate that this skin depth is increased by lowering the operating frequency and it is estimated as about 7 cm at 250 MHz frequency⁴⁹. Initially there was a fear of sensitivity loss by lowering the operating frequency, but recent experiments indicate that there would be no loss in signal for biological specimens. EPR images of nitric oxide measured at 700 MHz were recently reported by Yoshimura *et al.* measured in the head of a rat⁵⁰. Anatomic FT EPR images of mice were measured by

Muralikrishna *et al.* at NIH using a spectrometer operating at 280 MHz⁵¹. EPR images in three and four dimensions of a rat heart were measured at L-band frequency by Kuppusamy *et al.*⁵².

4 ENDOR

The unresolved hyperfine EPR spectrum leads to difficulties in identification of the nucleus and extraction of the hyperfine coupling constants. ENDOR technique resolves this problem by selectively exciting an electron spin transition and looking into the nuclear transitions from there. The resolution and specificity increase because of smaller number of ENDOR transitions and selectivity of EPR line. A spin $S=1/2$ system coupled to N inequivalent nuclei of nuclear spin $I = 1/2$ gives only $2N$ lines while an EPR spectrum would have 2^N lines.

A schematic diagram of ENDOR transitions is shown in Fig. 9. An ENDOR experiment requires a high power MW source to excite the electronic transition, an rf generator and a specially designed cavity since the NMR spectrum is measured at radiofrequency while the excitation of EPR transition takes place in microwave region. Pulsed EPR also prompted the development of pulsed ENDOR — the main advantage being exclusion of unwanted relaxation effects. Two pulse sequences were described in literature, one by Davies⁵³ and the other by Mims⁵⁴. Both are based on transfer of spin polarization. Davies ENDOR uses selective MW pulses while Mims ENDOR is based on measurement of stimulated echo. The pulse sequences of pulsed ENDOR are shown in Fig. 10. In Davies ENDOR, a single EPR transition is selected and its polarization is inverted using a π MW pulse. Then a selective π rf pulse is applied to match the NMR transition which inverts its polarization. Consequently, the population difference in allowed EPR transition would disappear. At this stage, a two-pulse echo is used to measure the change of polarization in EPR transition as a function of rf. The ENDOR line is indicated by disappearance of EPR signal. The Mims scheme is based on stimulated echo sequence. The first two π MW pulses cause a periodic polarization pattern in EPR transition while the π rf pulse inverts the NMR polarizations. Consequently, a change in polarization would be observed. This is measured in the stimulated echo

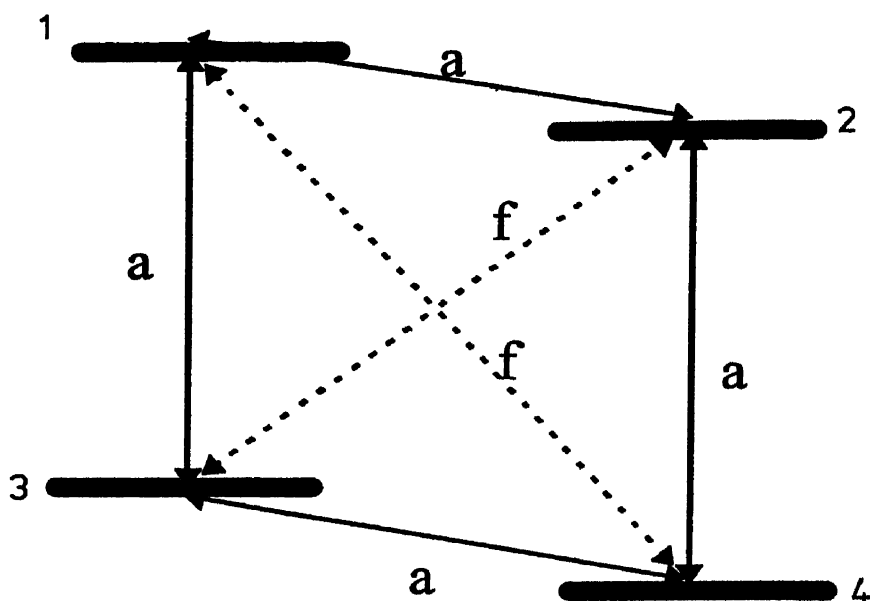
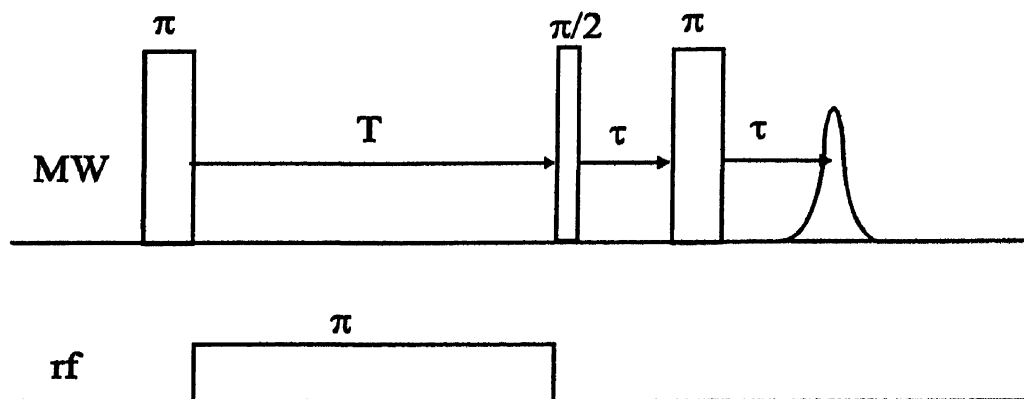


Fig. 9 Energy level scheme of ENDOR transitions *a*, allowed transitions; *f*, forbidden transitions

(a)

DAVIES



(b)

MIMS

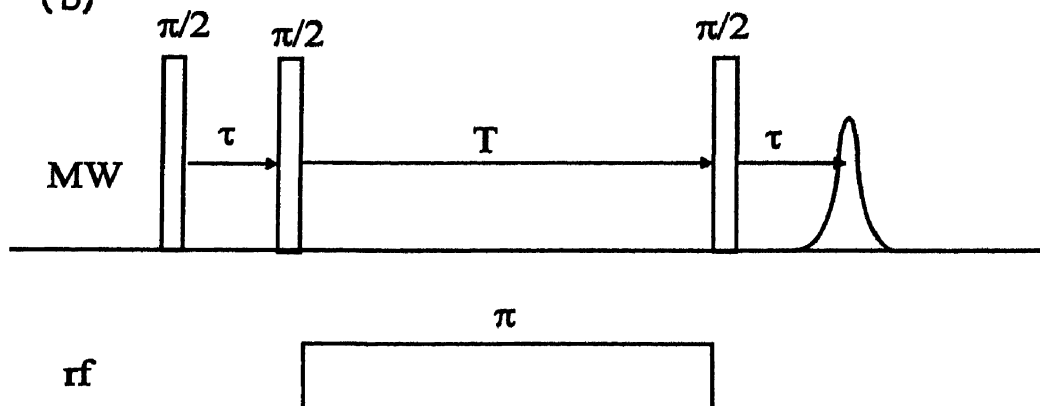


Fig 10 Pulsed ENDOR sequences. (a) Davies ENDOR sequence. (b) Mims ENDOR sequence

after third $\pi/2$ MW pulse. In Chirp time Domain ENDOR, the power broadening caused by the above techniques can be avoided. This technique uses rf pulses instead of monochromatic rf⁵⁵. It is often useful to combine ESEEM and pulsed ENDOR techniques while studying weak hyperfine signals. The weak hyperfine lines in ENDOR spectrum can be enhanced using ESEEM studies.

5 Mössbauer Spectroscopy

Mössbauer spectroscopy deals with the γ -ray absorption or emission spectra between nuclear states. For nuclei, the recoil energy is very large making the emission and absorption difficult. Rudolf Mössbauer discovered that for nuclei placed in solids at low temperature, there is a finite probability that emission or absorption of the γ -ray photon will take place without absorption or emission of a phonon⁵⁶. This means that the solid will be in the same internal state before and after the absorption or emission. The recoil is taken up by the crystal as a whole and not by an individual atom. Since the γ -ray absorption is recoil-free, it can be resonantly absorbed by the stationary atoms. It is possible to observe a resolution as small as 2.8 MHz by this technique. Usually, this resolution is good enough to for the measurement of nuclear hyperfine splittings.

The hyperfine energy levels are scanned by moving a radioactive source repetitively toward and away from an absorber. The γ -ray source is a radioactive element that is mechanically vibrated back and forth to Doppler shift the energy of the emitted γ -radiation. This varies the energy of the γ -rays arriving at the absorber. The detector records the frequencies of γ -radiation that are absorbed by the sample. ⁵⁷Co, which can be alloyed with Fe or Pd, is typically the source for ⁵⁷Fe Mössbauer studies.

Mössbauer spectroscopy is extremely useful for the determination of the oxidation state of iron compounds by means of the determination of their quadrupole coupling values. The compound will have a slightly different resonant absorption energy if it is in a different coordination state than the source due to the shielding of *d*-electrons. This results in isomer shift (or sometimes chemical shift). These chemical shifts are characteristic to the oxidation states and also valuable in estimation of quadrupole coupling⁵⁷

6 NQR Spectroscopy

Nuclear Quadrupole Resonance, is a zero-field technique to measure NMR. It is a valuable technique to observe the nuclei with spins $> 1/2$ such as deuterium, ¹⁴N, ³⁷Cl, ⁶³Cu, ⁶⁷Zn, ¹²⁷I. These nuclei have a nuclear magnetic dipole moment, which makes them NMR active. In addition, their electric quadrupole moment interacts with the gradient of the local electric field at the site of the nucleus, to give an electric quadrupole coupling. The size of this coupling depends on the strength of the nuclear quadrupole moment, field gradient at the nucleus, and on its Sternheimer factor which amplifies the field gradient that the nucleus feels. Often the electric quadrupole coupling is larger than the conventional NMR frequency, even for the largest magnets currently available. Thus NQR does not require a magnet to produce NMR signals. The internal field requires that the substance must exist in a crystalline state. Currently two or three-dimensional NQR pulse sequences are developed for the study of spectra of proteins and other molecules of biological interest⁵⁸.

7 Concluding Remarks

The CW era of magnetic resonance demonstrated the quantum mechanical phenomena of electron and nuclear spin properties with respect to molecular geometry and magnetic properties. The high sensitivity and measurement of weak interactions made it an elegant investigative and analytical technique. In the FT era, NMR has taken the direction of multidimensional studies, and imaging making it an important resource for research and medicine. EPR spectroscopy is progressing in the development of novel experiments in using pulsed techniques and biological applications. These are demanding development of special instrumentation, methodology and novel spin probes suitable for a variety of applications. *In vivo* EPR measurements of free radicals, paramagnetic species like oxygen and EPRI are emerging with promising results to transform it into a medical diagnosis and investigative technique in biology.

In principle, the FT EPR technique is faster than FT NMR by orders of magnitude because the relaxation times are much faster in EPR. EPR generally requires 10-20 ns pulses. Thus with suitable electronic switches and ultra fast signal

acquisition and digitization equipment, millions of EPR responses can be acquired in a second. This makes EPR at least five to six orders of magnitude faster than NMR. Thus a real-time imaging would be possible using EPRI. Using paramagnetic

probes of appropriate molecular weight and size, one can also target specific organs in the animal. With appropriate spin probes and strong gradients, spatial resolution of micron level is achievable by EPRI.

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NMR OF BIO-MACROMOLECULES

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Nuclear Magnetic Resonance has emerged during the last two decades as the single most powerful tool for determination of high resolution structures of biological macromolecules, in aqueous solutions under conditions akin to those prevalent in intracellular environments. The technique has seen radical developments, both with regard to physical concepts and with regard to instrument technologies. While the high magnetic fields have enabled greater dispersion of signals, heteronuclear multidimensional experiments have enabled spreading of the information in several dimensions making use of the different chemical shifts of the different nuclear types. This development has embraced molecular biology in all its glory for the preparation of isotopically labelled molecules in large quantities. Dealing with large amounts of data and extensive computer modelling calculations have encompassed software technologies as integral parts of NMR developments. Till date high resolution solution structures of more than 300 proteins and nucleic acid systems, having molecular weights in the range of 5-15 kDa, have been determined and the trend is to study even larger molecular systems and macromolecular complexes. The present review surveys the general trends with examples drawn largely from the work carried out in our own laboratory.

Key Words: Biomolecular Structure; Multidimensional NMR; Nucleic Acids; Proteins

1 Introduction

Nuclear Magnetic Resonance (NMR) and its applications in chemistry have been growing at an exceedingly rapid pace ever since its discovery in 1945^{1,2} by two groups of researchers led by Bloch and Purcell at Stanford University and Massachusetts Institute of Technology respectively. Among the variety of applications to which NMR has been put, molecular structure determination in solution media stands out prominently and continues to attract the attention of a large number of researchers world wide. Very high investments are being made for these purposes and it is a common thing to see dedicated spectrometers in individual laboratories, despite the high cost of these machines. The molecular size limits have gone up from 10-20 atoms in the early 1950's to 5000 atoms in the 1990's. The structural detail obtained by NMR today is comparable to that obtained by X-ray crystallography which has so far been the single reliable technique for molecular structure determinations. Several protein

and nucleic acid structures are being determined every year by both these methods and also with comparable speeds. Thus with the availability of two reliable and independent methods now, each one of them serves as a complementary technique for the other, for comparisons, starting models etc.

Several factors have contributed to the success story of NMR described above. New concepts and techniques have been discovered. These include, chemical shift, coupling constant, spin decoupling, pulsed excitation, spin echo, nuclear Overhauser effect (NOE), Fourier transform NMR, water suppression techniques, polarization transfers, two dimensional NMR, three dimensional NMR, triple resonance experiments etc., to name a few. In parallel, spectrometer designs and performances have improved enormously over the last three decades. The magnetic field strengths have gone up from 4-5 kGauss in the 1950's to 180 k Gauss in the 1990's. This implies an approximately 250 fold increase in the sensitivity and a 40 fold improvement in the resolution in the spectra. Superconducting magnets resulted in highly stable fields and contributed to enhanced resolution and

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better line shapes in the spectra. Along with these developments were the sophistications brought about by the introduction of computers in the spectrometers and the breath-taking speed in the progress of the computer technologies

In addition to the NMR methodology related developments, the new discoveries in the areas of chemistry and biology had their roles to play in the success story of NMR structure determination. Noteworthy among these are the genetic engineering methods, polymerase chain reaction (PCR) and new synthetic routes for peptide and nucleic acid synthesis. With the genetic engineering and PCR methods, it became possible to produce desired segments or domains of large proteins selectively and in large amounts necessary for structure determination experiments. It also became possible to isotopically label and enrich these molecules, which is a basic requirement for performing many of the present day double and triple resonance experiments. In a like manner the new synthetic routes enabled production of large amounts of pure nucleic acid and peptide segments, which paved the way for detailed structure characterizations and also provided the basic 'primers' in PCR reactions.

The present article is intended to be a trace of the excitements in the area of structural chemistry and biology by NMR. There is no intention of making it an exhaustive review and accordingly a number of examples are taken from the work carried out in our own laboratory. A few exciting illustrations are also taken from others research wherever necessary. The language and the style are deliberately maintained to be qualitative avoiding all the technical jargons of NMR, so that the material can appeal to the general readership. In the following we present a systematic description of the relevant methodological developments and this will be followed by their applications in different structure determinations. In the end we give a peep into the future following the current state of knowledge and thinking within the scientific circles.

2 Multidimensional NMR Methods

The invention of two dimensional (2D) NMR in the 1970's^{3,4} caused the second major revolution in NMR, the first being the Fourier transform NMR, in the 1960's. The basic idea of the technique was

to display the interactions (correlations) between the various nuclei in a given molecule on a plane, thereby enabling unambiguous assignment of the individual resonances to specific nuclei in the molecule. This in turn enabled quantification of the interactions as an essential step towards three dimensional structure determination.

Fundamentally, there are two types of interactions between nuclei which are of significance for structure determination by high resolution NMR. These are: (1) J-coupling interaction which is mediated by the electrons in the intervening bonds and (2) through space dipole - dipole interactions. These interactions cause magnetization transfers between nuclei whenever they are somehow perturbed and kept in a non-equilibrium state. The two kinds of transfers caused by the two types of interactions have distinctly different properties and hence can be separated and observed independently. This is the basis for the design of all two dimensional and higher dimensional NMR experiments.

The essential elements of one, two and three dimensional NMR experiments are sketched in Fig. 1. Conceptually, a two dimensional experiment is an extension of the one dimensional FTNMR scheme which is a time domain experiment. The data collected as a function of time following the excitation of the spin system, is Fourier transformed to generate an NMR spectrum. In the 2D experiment the above time axis is segmented by introducing additional time periods before data collection. In a generalized sense, the time axis is considered to be divided into four periods namely, Preparation, Evolution, Mixing and Detection, and the data is collected only during the Detection period. Likewise a three dimensional experiment can be conceived as an extension of a two dimensional experiment and can be treated as a combination of two 2D experiments. Higher dimensional experiments can be generated in a similar manner. In all the cases the time domain signal is actually collected during the Detection period only. Evolution periods are variable time periods in the sense that they are systematically incremented during the course of the experiment. Consequently, the time domain data is collected as a function of two or three time variables in 2D and 3D experiments respectively. Subsequent Fourier

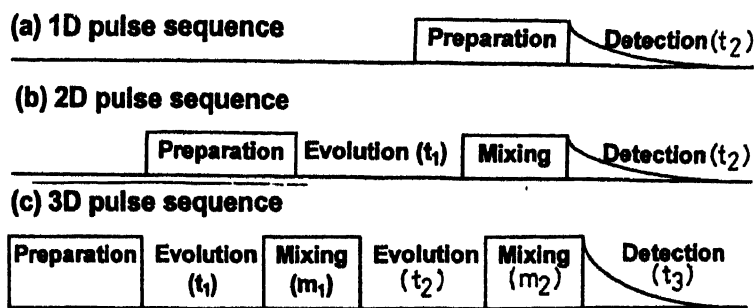


Fig 1 Schematic of one, two and three dimensional NMR pulse sequences. The time axis is segmented into various periods as indicated. The data is actually collected only during the detection period in each case. The evolution periods serve as indirect detection periods to generate the other frequency axes. Mixing periods help in causing magnetization transfers between the spins in the network.

transformation of these data with respect to each of the time variables generates the additional frequency axes of the 2D or 3D experiments. The mixing periods following the evolution periods help in causing magnetization transfers between nuclei which are frequency labelled during evolution and thus the detected signals carry information about the history of the spin system evolutions. Consequently the evolution periods serve as indirect detection periods of the experiment. The Preparation period is used to prepare the nuclear spin system in a suitable equilibrium or a non equilibrium state depending upon the purpose of the experiment. All of these periods may simply be delays or may contain radiofrequency (RF) pulses and these distinguish the various 2D and 3D experiments.

The 2D, 3D and other higher dimensional experiments can be classified as Homonuclear or Heteronuclear, according to the types of nuclei being correlated in the experiment. In a homonuclear experiment all the frequency axes belong to the same type of nuclei, for example, proton. In contrast, in a heteronuclear experiment, one or more of the frequency axes necessarily belong to a different type of nucleus or nuclei. In most cases, the detected nucleus is ^1H for sensitivity reasons. What this means is that the frequency information of the heteronuclei evolving during the 'evolution' periods is somehow transferred to the ^1H for detection. There exist a number of approaches^{5,6} for such 'polarization transfers' and the most commonly used ones are INEPT (Insensitive Nuclei Enhancement by

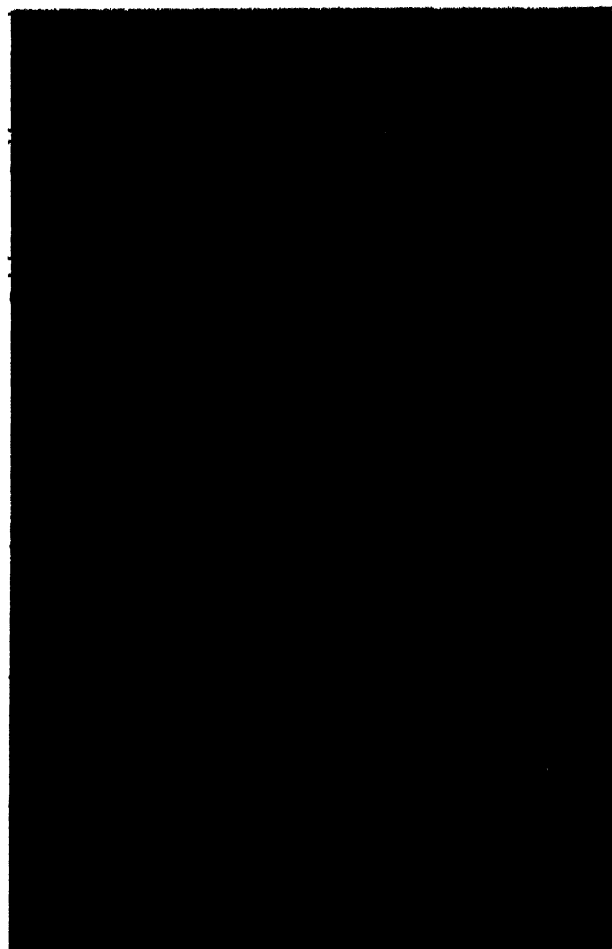


Fig 2 Progressive increase in the complexities of the experimental pulse sequences as we go from the simplest 1D FTNMR⁵¹ experiment through a 2D NOESY⁵² pulse sequence to a 3D triple resonance HNCA⁵³ experiment. Each vertical thick bar represents a radiofrequency pulse. The magnetization transfer pathways in each case are indicated by coloured lines. Clearly the triple resonance experiments have many more pulses and other parameters to be optimized and demand greater stability and performance from the spectrometers. They require three independent channels. The last row in the sequence is for gradient pulses which are used for selection of transfer pathways and also for suppression of unwanted signals.

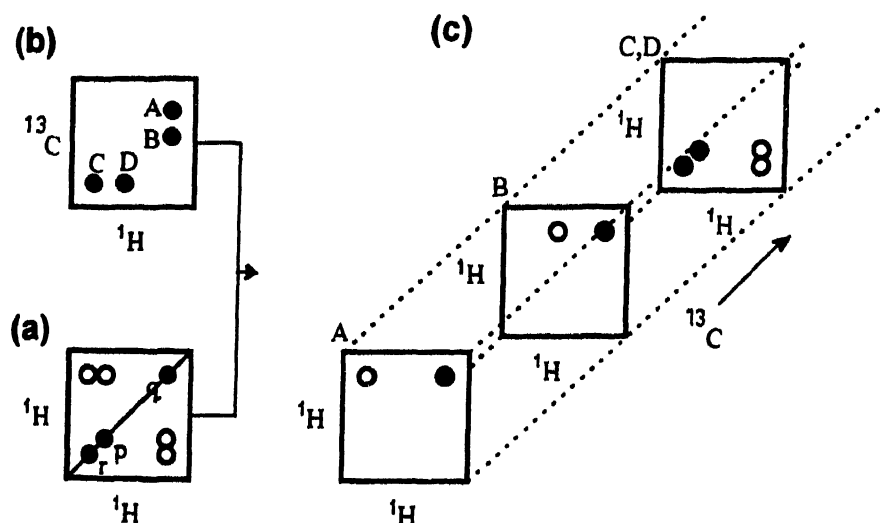


Fig 3 Schematic 2D ^1H - ^1H (a), ^1H - ^{13}C (b) and 3D ^1H - ^1H - ^{13}C (c) spectra to show their relative advantages and disadvantages. Ambiguities of assignments in 2D spectra can be resolved from the 3D spectra (see text)

Polarization Transfer), DEPT (Distortionless Enhancement by Polarization Transfer), and cross polarization by Hartmann-Hahn transfer. Fig. 2 illustrates the progressive increase in the complexity of the experimental sequences as we go from the simplest 1D experiment to a typical 3D heteronuclear triple resonance experiment.

The information content and characteristic features of common 2D and 3D spectra are schematically shown in Fig 3. Panel 'a' is a homonuclear ^1H - ^1H 2D correlated spectrum while panel 'b' is the hypothetical ^1H - ^{13}C spectrum of the same spin system designed to show the advantages of the heteronuclear spectrum in resolving the ambiguities in the ^1H alone spectrum. Each off-diagonal peak in 'a' represents a ^1H - ^1H correlation as indicated by horizontal and vertical lines. However an ambiguity arises in specific identification of the peaks at 'q' because of identical F1 chemical shifts. It remains uncertain as to whether there are two protons at 'q' coupled to two different protons or whether there is only one proton coupled to two different protons. This is resolved in panel 'b' because of the fact that at the particular proton chemical shift two carbon chemical shifts are correlated. A converse situation arises for the other two protons for which the carbon chemical shifts are identical. Thus a combination of both these experiments would help resolve all the ambiguities. This is illustrated in the

3D spectrum shown in panel 'c' which has been generated by combining the spectral features of the two spectra in panels 'a' and 'b'. In Figs 4 and 5 are shown illustrative homonuclear and heteronuclear, and two and three dimensional NMR spectra of some large biomolecules recorded in our laboratory. The advantages of a 3D ^{13}C resolved TOCSY spectrum over the 2D ^1H TOCSY spectrum are clearly evident.

The information content of a multidimensional NMR spectrum is determined by the pathway of magnetization transfer among the spins through the pulse sequence of the experiment. This is illustrated in the 3D pulse sequence in Fig. 2 by the dashed line. The transfers could be mediated either through the J -couplings or through the dipolar couplings between the various spins. The former is referred to as coherence transfer and the latter which results in incoherent transfer is also often described as NOE (nuclear Overhauser effect) transfer. In a particular pulse sequence there can be several steps of coherence transfers and these may also be combined with NOE transfers. While the different pathways of coherence transfer help in filtering the spectra differently, a combination of these with NOE transfer helps in studying interactions between molecules. Separation of NOE interactions according to chemical shifts of carbon, nitrogen etc is an essential step in the study of large proteins from the structural point of view.

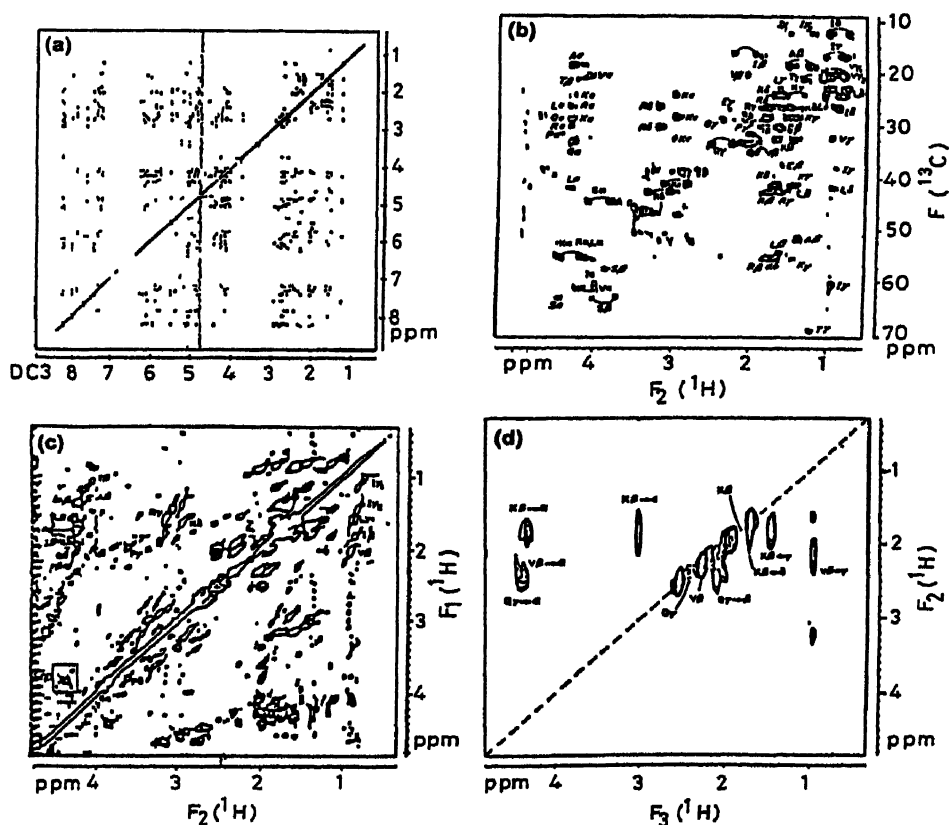


Fig 4 (a) 2D NOESY spectrum of 14 mer DNA segment (b) 2D TOCSY relayed ^1H - ^{13}C HMQC⁵⁴ spectrum of DNA binding domain Myb protein (Mol Wt 20 kD) (c) 2D TOCSY⁵⁵ spectrum of the same protein as in 'b' (d) A slice through the 3D ^{13}C resolved ^1H - ^1H TOCSY spectrum of the same protein⁵⁶ The slice represents a filter through the 2D spectrum shown in 'c' All the spectra have been recorded on a 500 MHz Bruker NMR spectrometer

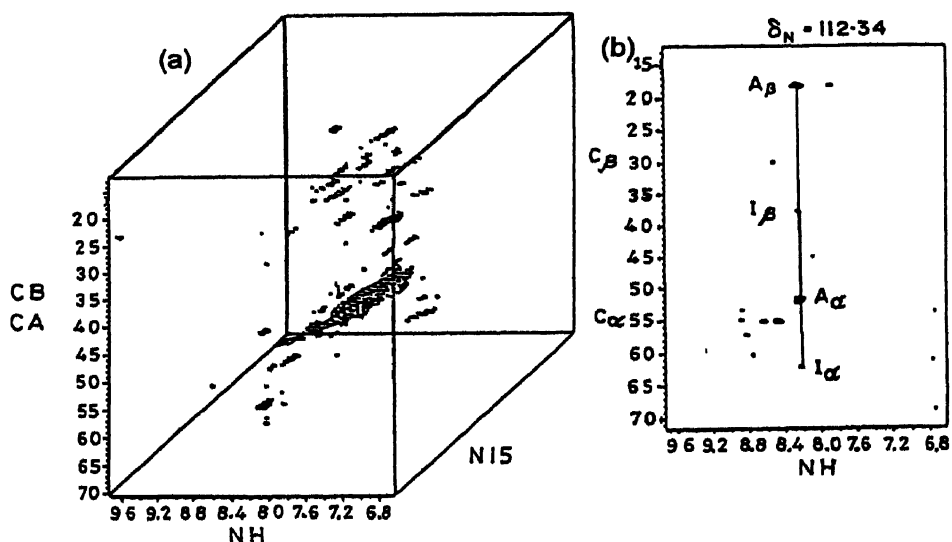


Fig 5 (a) A 3D CBCANH⁵⁷ spectrum of the same protein as in Fig 4 recorded on a 600 MHz Varian NMR spectrometer. This contains correlations between NH protons, ^{15}N of the i th residue and C^α and C^β nuclei of the i th and $(i-1)$ th residues along the amino acid sequence of the peptide chain (b) a slice through the spectrum in 'a' indicating the sequential connectivity between two residues (an Ileu-Ala stretch) in the polypeptide chain

With these ideas a large number (more than 100) of experimental sequences have been published to date. Several reviews and books have been written covering the principles of these techniques and also their applications to proteins, nucleic acids, and various types of biomolecular complexes⁵⁻¹⁰

3 Isotope Labelling of Proteins and Nucleic Acids

The heteronuclear multidimensional experiments on proteins depend crucially on efficient transfer of magnetization from ^1H to the NMR active isotopes ^{13}C and ^{15}N and transfer back to the proton for observation. At natural abundance these nuclei are available only to the extent of 1.1 % for carbon and 0.37 % for nitrogen. That means, the magnetization from only those protons attached to these nuclei will participate in the coherence transfer processes. This will clearly throw away a major portion of the proton magnetization and the sensitivity of the experiments will be extremely low. Besides, transfers between two heteronuclei would be unthinkable in such a situation; consequently complex experiments with multiple transfer steps involving heteronuclei would become formidable. Therefore, for the success of the above experiments it is absolutely necessary to have molecules with uniform isotopic enrichments. For most experiments enrichment to the level of 100 % is essential, but for some special situations partial enrichment to the extent of 50-70 % may be desirable.

Labelling by ^{13}C and ^{15}N in proteins and nucleic acids opens up possibilities of measuring heteronuclear one, two and three bond coupling constants. While, on the one hand this knowledge is crucial for the design of the experimental sequences, the three bond coupling constants are also intimately related to specific torsion angles in the molecules, on the other. These provide important and otherwise unavailable structural inputs. A substantial effort has therefore been devoted in recent years to measurement of such coupling constants in large proteins and nucleic acids and these have complemented the structural informations derived from the NOE data

In addition to ^{13}C and /or ^{15}N labelling, many a

times ^2H labelling has often been used in proteins. If all the nonexchangeable protons such as those on the α -carbons and those in the side chains are deuterated, the only signals observable in H_2O solutions will be the exchangeable protons such as the amides in the protein. Deuteration also eliminates one of the efficient relaxation mechanisms namely, ^1H - ^1H dipole-dipole relaxation and thus causes a significant narrowing of the amide signals. This not only helps in increasing the resolution in the spectra but also permits design of experiments with longer pulse sequences. Because of the longer relaxation times of the protons the signal survives longer along the time axis and thus more steps of magnetisation transfers can be included in the pulse sequences. The disadvantage of deuterium labelling, however, is that it leads to loss of information which is contained in the different ^1H - ^1H interactions. Thus it will never be possible to obtain a complete 3D structure of a protein by a fully deuterated protein alone. Deuterium labelling may also cause small changes in the chemical shifts of protons and other nuclei. Thus deuterium labelling is always used as an ancillary strategy for resolving ambiguities due to signal overlaps and often partial labelling is used to circumvent some of the problems mentioned above.

While uniform enrichment of all the carbons and nitrogens is useful for heteronuclear multidimensional experiments, selective labelling of a few amino acids or a group of amino acids in a large protein can be used as a selective filter. This way the amino acids located at the active site of an enzyme or at specific binding sites in a protein can be labelled and their signals can be monitored for understanding reaction mechanisms or mechanisms of specific recognition by proteins. Labelling of specific amino acids simplifies the spectra - of course by throwing away some information - and renders them easier to analyze. Quantitative analysis of the signals coming from such residues which may be spread over the whole length of a protein sequence, can help in studying protein folding pathways in large proteins.

The most common methods for isotopic enrichment of proteins and nucleic acids rely on biological techniques, namely, gene cloning, subcloning and protein engineering. The desired

proteins are produced by specially designed expression systems which carry the genes coding for the desired amino acid sequences of the proteins. The cells harboring these expression systems are grown in nutrient media containing isotopically labelled nutrients such as ^{13}C -labelled glucose and ^{15}N labelled ammonium chloride. Consequently, the expressed proteins will be uniformly isotopically enriched. Generally the expression systems are constructed in such a way that the desired protein is the major protein expressed in the cells. For deuterium labelling the cells will have to be grown in media prepared in $^2\text{H}_2\text{O}$ and all the nutrients supplied externally must be deuterium labelled. Similar strategies are used for production of labelled nucleic acids which employ *in-vitro* transcription systems.

Cloning, expression and labelling of proteins appears straightforward in principle, but is often beset with several uncertainties^{11,12}. First of all, for a new protein, it is difficult to predict the efficacy of the expression system used. The expressed protein may turn out to be harmful to the cells and then the cells may die. The growth of the cells in isotopically enriched media - so called minimal media - is usually slower than in rich media with normal nutrients. There could also be problems of post translational modifications of the expressed proteins by the cellular processes. The expressed proteins may get associated with other proteins inside the cells and then their structures may change or it may become difficult to extract the proteins. When the cells are grown in $^2\text{H}_2\text{O}$ for deuterium labelling, the growth rates are much lower and some times may not even grow. The cells will have to be slowly adapted to the new solution conditions by progressively increasing the $^2\text{H}_2\text{O}$ content in the media. Therefore, it is clear that a lot of care has to be taken in designing the expression systems and even so, a fair amount of trial and error is involved in successful production of labelled and functionally active proteins.

4 Computational Techniques in NMR Structure Determination

Analysis of multidimensional NMR data, as will be described in the following sections, provides two types of structural inputs, namely, interproton distances and torsional angles. Then one has to

construct molecular structures which satisfy these inputs treating them as some kind of constraints. An important consideration here is that the conformational space which is multidimensional has to be appropriately scanned so as to be sure that the derived structures are unique. It is also necessary to ensure that the structure derived has the lowest conformational energy and truly represents the global minimum on the potential energy surface. This becomes a particularly formidable task when the inputs derived experimentally are rather limited. Further, one has to see the effects of starting models and the effects of the environment on the structures derived. These considerations have led to the development of two types of algorithms which are briefly discussed below for the sake of completeness.

Distance Geometry

This is a general method for converting a set of distance bounds between atom pairs into a configuration of these atoms that is consistent with the bounds¹³. In this algorithm, a molecular structure is described in terms of the set of all pairwise interatom distances which is written in the form of a so-called 'distance matrix'. For a correct structure the experimentally derived distances must figure in such a matrix as precisely as possible. This is achieved by defining a 'target function' which represents the violations from the distance inputs of the actual distances in a structure. The target function is minimized so as to drive the structure towards best satisfaction of all the constraints. The tolerance in the target function satisfaction determines the precision of the structure.

The above algorithm has been used successfully in determining structures of several proteins and nucleic acid molecules. However one of the difficulties in this approach is that the minimizer has to spend a lot of time and effort in satisfying the covalent geometry of the molecule which is conformationally invariant. To circumvent these problems algorithms have been devised which work in the torsion angle space whereby, directly bonded atom distances and bond angles are automatically fixed^{14,15}. These algorithms are currently most popular because of their speed which consequently enables a better search through the conformational space.

Molecular Dynamics and Simulated Annealing

The distance geometry protocol does not explicitly include the energy of the system as a variable and thus does not ensure that the structure derived as an acceptable structure has the lowest possible conformational energy. This problem is overcome in the 'Molecular Dynamics' protocol¹⁶. An energy function including all different interactions is defined and the Newtons equations of motion are solved numerically to derive the trajectories of atomic motions as a function of time. These reflect, on one hand the atomic motions and help to identify the stable states in the system, on the other. From these states, energy minimization relieves the strain present in the system and helps finding the local minima on the potential energy surface. Molecular dynamics calculations allow potential barriers to be crossed, and thus enable identification of several minima on the potential energy surface. In simulated annealing the molecule is heated to a very high temperature and then slowly cooled to the temperature at which molecular dynamics calculations are to be carried out. This protocol allows a better search through the conformational space and thus can help in locating the global minimum more reliably. This also can generate several starting models for dynamics and eliminates the biases of the starting models on the final structures.

Molecular dynamics calculations can also be carried out in the presence of various types of constraints given in the form of suitable potentials. This restricts the conformational space to be searched and speeds up the computations. Further, if these constraints are derived from experimental NMR data in aqueous solutions, then the structures represent truly the solution structures of the molecules.

5 Nucleic Acid Structure and Dynamism

DNA and RNA, the two types of naturally occurring nucleic acids, are polymers consisting of a repeating sugar-phosphate backbone in which, to each of the sugar rings is attached one of the four bases, adenine (A), thymine (T) (or uracil U), cytosine (C) or guanine (G). DNA and RNA differ basically in the type of the sugar ring (deoxy ribose in DNA and ribose in RNA) and in that thymine in DNA is replaced by uracil in RNA. However,

structurally and functionally, the two types of nucleic acids are widely different. RNAs exhibit a wide variety of folded structures compared to DNA and are also directly involved in a variety of functions. DNA, on the other hand is the genetic material which exists largely as a duplex wherein two polymeric chains or strands are held together by base pairing; A pairs with T with two H-bonds and G pairs with C with three H-bonds. The sequence of the bases in each strand is the genetic information which is expressed in a regulated manner by the proteins carrying out the variety of functions in the cell.

NMR spectroscopy has contributed immensely during the last two decades to our present knowledge of nucleic acid structure and function inside a cell. Atomic level details of three dimensional structures of several DNA duplexes have been determined and these have indicated that the duplex is not a regular structure as had been thought before, but rather is highly irregular with important sequence dependent variations. Thus the backbone of the DNA has some sort of a signature of its sequence and this is what enables some enzymes such as restriction enzymes and proteins such as transcription factors recognize specific sequences in a long million base DNA. Besides, the story has gone much beyond the DNA duplex which caused a major revolution in the 50's and led to the birth of the new area in science namely, 'Molecular Biology'. Although it was believed that DNA can form many other different kinds of structures, either transiently or in the presence of some other agents, a detailed characterization of several unusual forms has been achieved only during the last two decades. We will present below the NMR derived structures of these unusual forms after giving a short description of the NMR strategy of structure determination.

NMR Strategy of Nucleic Acid Structure Determination

Predictably, the first step in all such efforts is to obtain resonance assignments for all the protons in the molecule. Subsequently the interactions between the assigned protons must be quantified to derive the structural informations. By and large, nucleic acid structure determination has relied on homonuclear ^1H - ^1H 2D correlated experiments, although occasionally, heteronuclear experiments employing ^{31}P have also been used. In nucleic

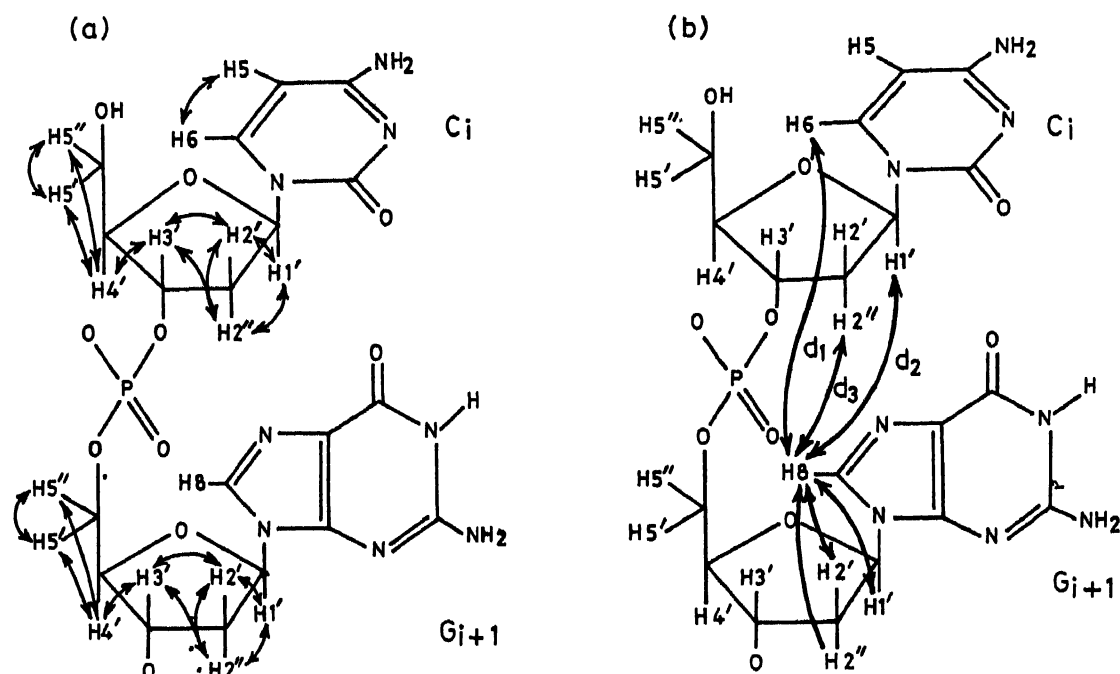


Fig 6 Observable J -correlations (a) and NOE correlations (b) in a duplex DNA segment Together, these enable complete sequence specific assignments in DNA segments

acids, two types of protons can be easily distinguished. These are the exchangeable imino protons which resonate in a distinct region between 11-16 ppm downfield of the reference TSP (tetramethyl silyl propionate) and the non-exchangeable protons which resonate between 1-9 ppm downfield of TSP. The former are direct monitors of base pairing and indicate the symmetry, strand orientations etc.; the latter are useful for detailed characterization of the structures.

Fig. 6 shows the observable correlations in nucleic acids in 2D NMR spectra^{9,17-19}. Panel 'a' displays the correlations mediated by J -coupling interactions between protons while panel 'b' shows the NOE correlations. The J coupling correlations help in identifying spin systems of the individual nucleotide units and the NOE correlations permit a sequential walk along the length of the molecule. Using these two types of correlations it is straight forward to obtain sequence specific assignments for all the protons in a given nucleic acid segment. An important point to note here is that most of the NOE correlations between nonexchangeable protons observed would be between adjacent nucleotide units in the same strand. This helps a great deal in obtaining specific assignments.

After the assignments, the NOE interactions are quantified to extract interproton distances by making use of the fact that the intensities under certain conditions are inversely proportional to the sixth power of the respective interproton distances. Thus the NOE intensities are a sensitive function of their distances and from experience it is generally observed that the observation of a NOE correlation limits the distance to less than 5-6 Å. These interproton distances constitute the major input for computational algorithms such as restrained Molecular Dynamics or Distance Geometry for reconstruction of the three dimensional structures of the molecules. The conformational space which is multidimensional is searched for identifying all those conformations which satisfy the experimental interproton distance constraints, torsion angle constraints derived from J -correlated spectra and also some other constraints regarding base pairing, symmetry etc. Because of the uncertainties in the estimation of the constraints, there is always a tolerance specified and at the end of the conformational search, one ends up with a family of closely related structures. For each structure, the NOE intensities are calculated by rigorous relaxation matrix methods^{20,21} and then the structures are screened on the basis of quantitative fitting of calculated and experimental NOE

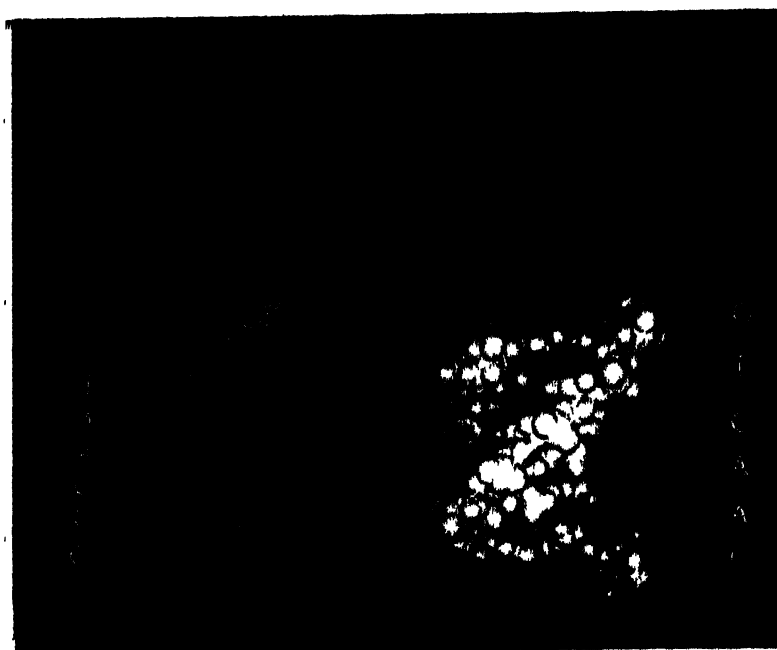


Fig 7 Solution structures of two DNA sequences ACCGTTAACGGI and ACAACTGCAGTTGTT determined by NMR in our laboratory^{22,23}. The space filled regions are the Myb cognate sites in the two molecules. Rest of the portion is shown in stick model to indicate the base pairing and the backbones explicitly.

intensities. It is generally observed that larger the number of the constraints, better is the convergence of the structures and that the derived structure will also be unique. Nonetheless, depending upon the extent and distribution of the constraints defining different portions of the molecules, the structures will be defined to different levels of precision at different portions. The NOE intensities also carry information regarding the dynamism in the molecules and a proper quantitative interpretation of the intensities reflects on these aspects as well.

Polymorphism in DNA

(a) Duplexes

This is the most common form of DNA formed by holding together of two strands by specific base pairs A:T and G:C. Fig. 7 shows the atomic resolution structures of two DNA duplexes, 12 and 14 units long, determined in our laboratory^{22,23}. Fig. 8 shows an analysis of the local helical axis parameters in the two cases to illustrate the sequence dependent variations in the structures. Both these DNA segments contain recognition sites for the Myb protein, an oncogene product, but the recognition sequences have different flanking units and have different lengths of DNA on either sides.

These factors seem to cause substantial differences in the three dimensional structures of the two molecules.

(b) Mismatches

Base pair mismatches in DNA occur due to limited fidelity of the DNA polymerase enzyme and these are then corrected by the repair machinery of the cell. Sometimes, however, the mismatches go uncorrected for a variety of reasons one of which may be that the mismatches go unnoticed, which in turn reflects on the structural features at the mismatch sites. Some mismatches are tolerated more than others indicating that all the mismatches do not distort the duplex structure to the same extent. What would be the consequences of such mismatches in specific recognition processes? We have investigated this problem in one particular case namely, that of LFB1 recognition. This homeodomain protein recognizes the sequence TTAATNATTAA where N is any nucleotide unit. We have studied the structural consequences of GG and CC mismatches at the N site in the molecule²⁴. We observed that CC mismatch produces a looped structure with substantial distortion in the centre. On the other hand a GG mismatch can produce a variety of

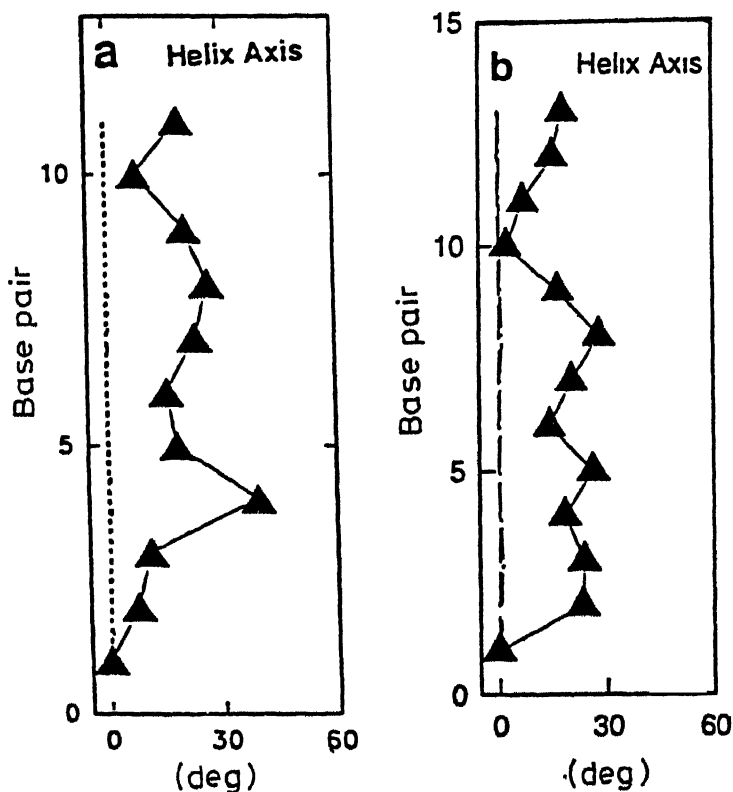


Fig 8 Progress of the helical axes in the structures of the two molecules shown in Fig 7. The zig zag path indicates the irregularities in the structures and these are different in the two molecules.

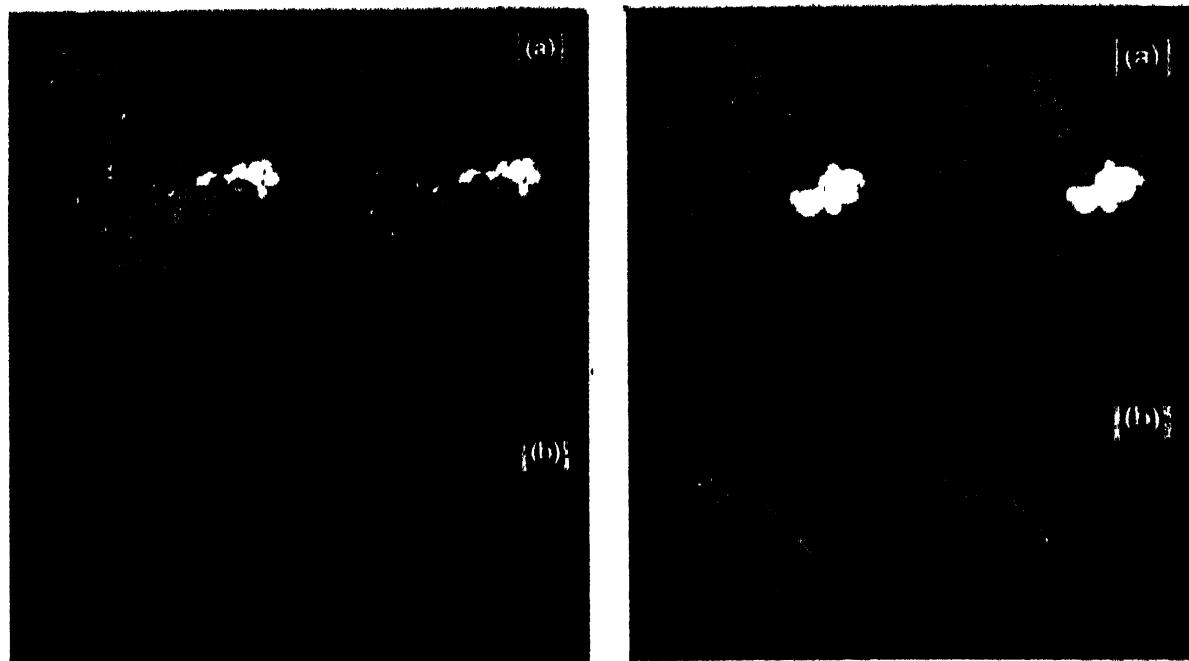


Fig 9 Structures of the sequence GTTAATGATTAAC which has a GG mismatch in the center as determined by NMR and MD simulations in our laboratory. The structure with a GG pairing (left) has slight distortions as compared to the normal B-DNA duplex, whereas the structure (right) without GG pairing shows a substantial bending and distortion in the center. Space filling models are shown on the top (a) while base pairing at the mismatch site is shown at the bottom (b).

distortions depending upon whether the two G's are paired or otherwise. A particular type of pairing causes minimal distortion in the duplex structure and such a structure may be tolerated by the LFB1 protein. Fig. 9 shows two different topologies that could be produced by a GG mismatch at the N site. It is also clear that the mismatches cause substantial dynamism in the molecules.

(c) Hairpins and Dumbbells

The genomic DNA which is a duplex has to open up for most of its functions. Then the nascent single stranded DNA can in principle undergo a number of structural transitions. Hairpins and

Dumbbells belong to this category of transient structures. Fig. 10 shows the structure of a DNA dumbbell determined in our laboratory recently²⁵. The stem of the dumbbell has two nicks in the center, yet it is noticed that the stem retains a good duplex structure with excellent stacking of the adjacent base pairs. The loop portion of the dumbbell exhibits a high degree of dynamism and dumbbell structures can be formed with different relative orientations of the loops.

(d) Triplexes

These are three stranded structures formed by two pyrimidine (T and C) strands and one purine (G and A) strand or by two purine strands and one pyrimidine strand. The base pairing is highly

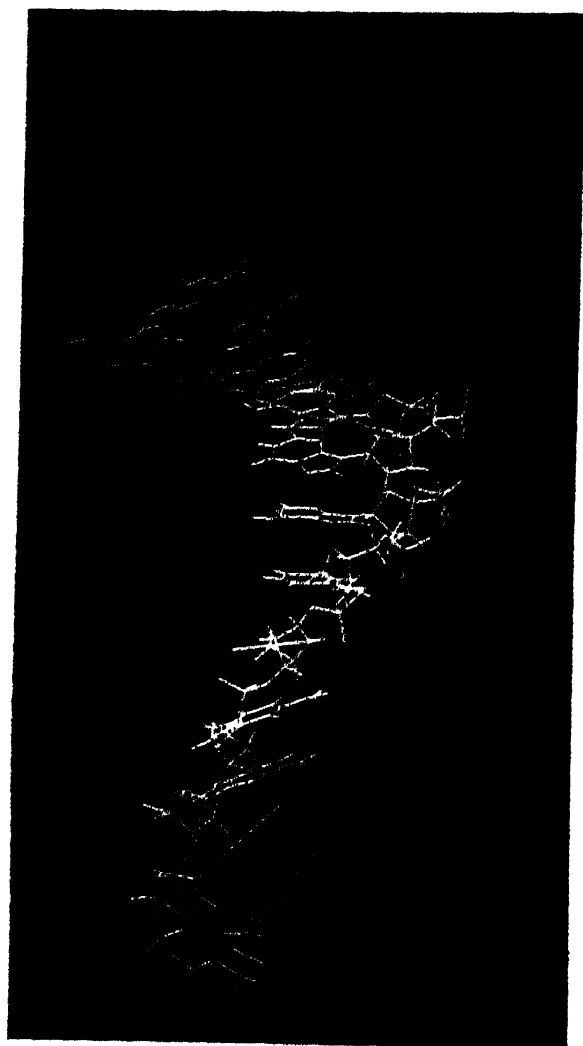


Fig.10 Solution structure of a DNA dumbbell formed by association of two molecules of the sequence GATCTTCCCCCGGAA₂ as determined by NMR in our laboratory²⁵. The stem of the molecule has two nicks in the middle, but the stacking of the base pairs is maintained through the nicks

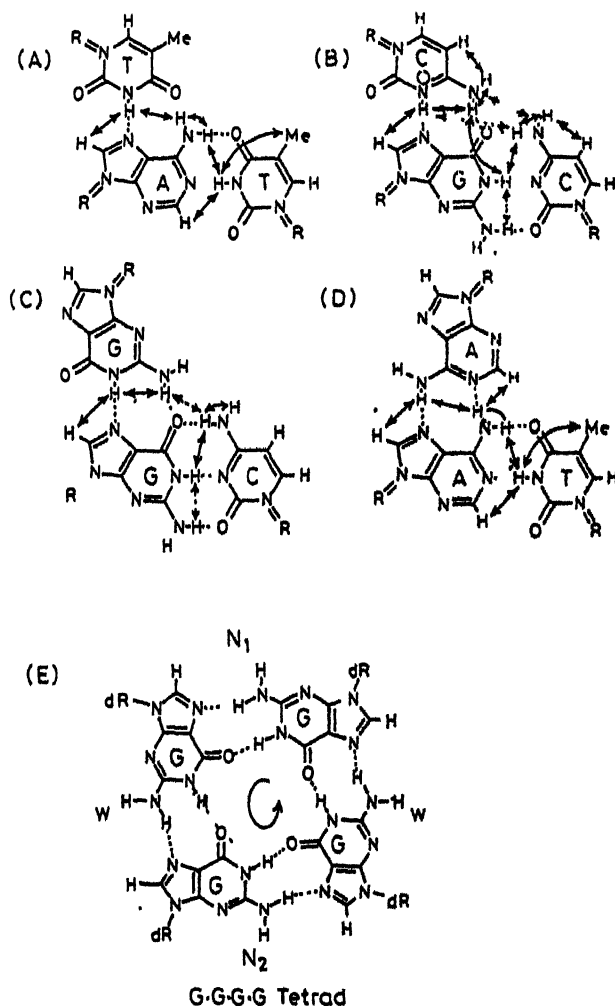


Fig 11 (A-D) Base pairing schemes in py.py.py and py.py.pu types of triplexes where py represents a pyrimidine base and pu represents a purine base. The arrows indicate the short distances observable by NOE in the NMR spectra (E) Base pairing scheme in the GGGG tetrad in G-quadruplexes.

specific and consist of T.(A T), C⁺ (G:C) triplets in the former type of triplexes and A (A:T), G (G:C) triplets in the latter type of triplexes (Fig 11 A-D)²⁶. In either case the like strands run in opposite orientations. In certain situations some mismatch triplets such as G (T.A) have also been observed. Fig. 12 shows the atomic resolution structure of one particular triplex molecule reported in the literature²⁷. It is seen that the triplex structure is formed by placing one strand (the third strand) in the 'major' groove of the duplex formed by one purine strand and its complementary pyrimidine strand, and the three bases in the H-bonded triplet lie almost in the same plane. H-bonding is very specific and these aspects play important roles in specific recognition and have applications in gene targeting and gene therapy²⁸. Triplex formation is also known to be playing a dominant role in recombination reactions. The structural details obtained by NMR provide a molecular rationale for these phenomena.

(e) G-Quadruplexes

It is known that the ends of chromosomes (so

called telomeres) have G-rich sequences which are believed to be playing important roles in cell differentiation and growth. The base G has the unique property that it has two donor sites and two acceptor sites for H-bond formation. As a result it can form a very stable H-bonded network with four G bases all in the same plane (so called G-tetrad), and this is what leads to the formation of four stranded structures involving exclusively G-tetrads stacked over each other²⁹⁻³². The H-bonding network in a G-tetrad is shown schematically in Fig 11E. Recently it has been observed that GCGC tetrads can also be formed in some situations although this network will have fewer H-bonds and the structure will be relatively less stable³³. Fig. 13 shows an illustrative G-quadruplex structure and the different ways such structures can be formed is schematically indicated in Fig. 14. The four strands can have different orientations and the bases in each strand can also have different orientations with respect to the sugar rings. Clearly there is a great variety in these structures and these must have important roles to play in recognition by enzymes such as telomerases, polymerases etc.

(f) i-motif

This is also a four stranded structure but is



Fig 12 Solution structure of a DNA triplex determined by NMR (reproduced with permission from ref. 26) The structure is shown both at high resolution and at gross level with ribbons and planks for better clarity of base and backbone orientations. Two orthogonal views are shown



Fig 13 Solution structure of a G-quadruplex determined by NMR (reproduced with permission from ref. 29). For the sake of better clarity, the structure is drawn with planks and ribbons to represent bases and backbone respectively. Different views are shown

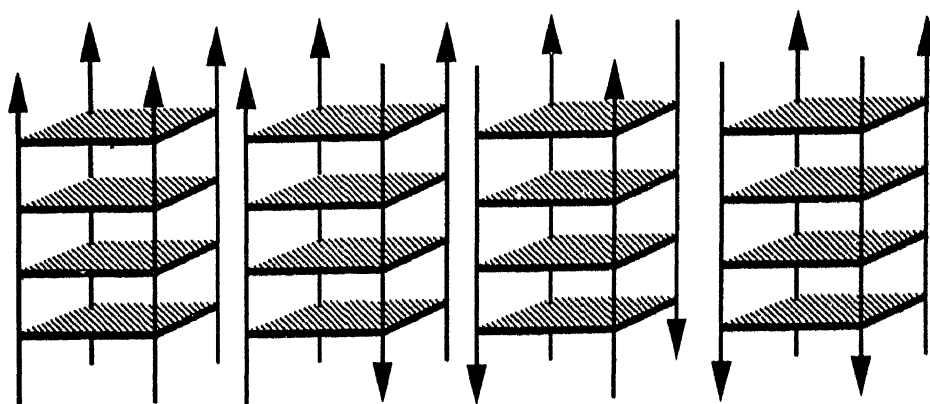


Fig 14 Schematic drawings of different possibilities of G-quadruplex formation. Each vertical line represents a G strand and the plane between the four strands represents a GGGG tetrad. The arrows indicate the orientations of the strands.

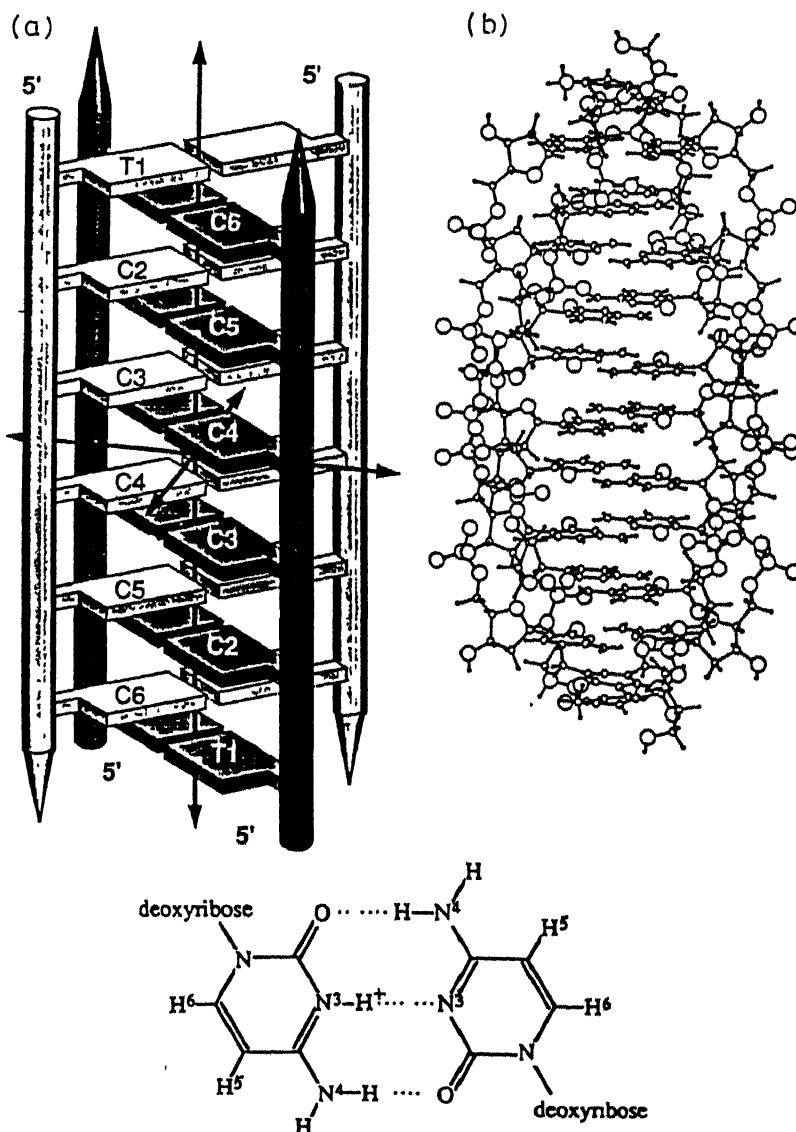


Fig 15 Schematic representation (a) and solution structure (b) of an i-motif formed by a C-stretch as determined by NMR. It consists of two parallel stranded duplexes interdigitating each other. The duplexes run in opposite directions. The C⁺-C base pairing responsible for the duplex formation is also indicated (taken from ref. 34).

formed by continuous C-stretches under low pH conditions³⁴⁻³⁶. Two C-strands running in parallel orientation interact via C-C⁺ pair formation and two such duplexes running in antiparallel direction interdigitate. The base pairing scheme and the NMR structure of the TC₅ sequence in which this structure was discovered are shown in Fig 15. Both the duplexes are stretched and unwound significantly and the strands look fairly extended. Again, this kind of structures are expected to be formed at the chromosomal ends of the DNA. The low pII required for the formation of these structures may be produced by the charged groups in the proteins binding to the DNA.

In addition to the above distinct structural motifs which have been investigated extensively by several researchers, there have been occasional investigations on many other types of structures such as three way, four way and five way junctions, cruciform DNA, parallel stranded duplexes, RNA-DNA hybrids etc. (reviewed in ref 8). Thus not only that the DNA exhibits the various above type of structures, it is also observed that depending upon the sequence and experimental conditions, a particular DNA can undergo structural transitions. A classic case has been noted in our laboratory during the last few months. The molecule with the sequence 5'-GATCTTCCCCCGGAA exists as a monomeric hairpin at low concentrations, forms a dumbbell with antiparallel duplex in the stem at higher concentrations, and goes over to a parallel stranded duplex and an i-motif structure as the pH is lowered. Thus it is clear that a given DNA segment can exhibit dynamism even at the topological level and these can be induced by the proteins in a living cell.

The number of investigations on RNA is relatively small because of practical difficulties, but currently, this is a hot area of research. Different RNA aptamers, hammerhead RNAs, tRNAs with folded structures are being investigated by multidimensional NMR experiments. Because of the greater complexities of the RNA spectra, isotopic labelling is becoming essential and heteronuclear NMR spectra are being used to resolve the spectra in different dimensions (see reviews in ref 8).

6 Protein Structure and Dynamism

Proteins constitute the most important component of the machinery of life. They are also the most complex molecules with greatest diversity in their sizes, properties, stabilities, structures etc. Consequently these are also the most widely studied molecules by a variety of physical and chemical techniques to unravel the secrets of their variety and specificities of functions. It is common knowledge now that proteins are made up of 20 different types of amino acids, and their sizes range from 50 to several thousands of amino acids in a single polypeptide chain. The sequence and composition of the amino acids largely determines the properties of a protein in terms of its 3D structure, behaviour under different experimental conditions, dynamism etc. Some proteins are made up of domains which function independently and this allows dissection of the domains for studying their properties.

The three dimensional structure of a protein molecule has well defined structural elements - so-called secondary structure elements - such as α -helices, 3_{10} helices, β -sheets (parallel and antiparallel), different types of turns, loops etc. Spatial arrangement of these units determines the 'tertiary structure' of the molecule. Each one of these elements is characterized by specific combination of torsion angles along the backbone, and in the case of helices and sheets there are also specific patterns of H-bonds between the amide protons and the carbonyl oxygens of the backbone chains. There are two torsion angles, namely, ϕ and ψ for every residue, which determine the fold of the polypeptide chain. The acceptable ranges of these angles from the point of view of avoiding short contacts and their ranges for specific secondary structures are described elegantly by the Ramachandran plots³⁷.

NMR Strategy for Protein Structure Determination

From the NMR point of view, a protein with 50 amino acids is a small protein and a protein with 300 amino acids is currently a large protein. The NMR strategies for protein structure determination have been evolving and the size limits may be pushed further in the years to come. While NOE remains the crucial and the final experimental input

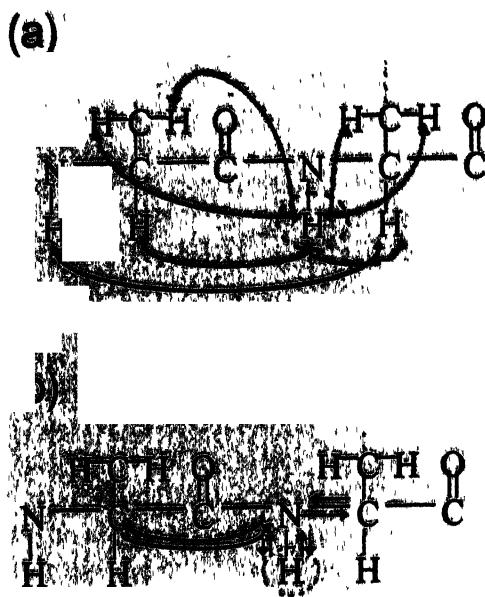


Fig 16 Strategies for obtaining sequence specific assignments in small (a) and large (b) proteins. The former rely on use of ^1H - ^1H J and NOE correlated techniques while the latter make use of triple resonance experiments involving ^1H , ^{13}C and ^{15}N correlations. The arrows in 'a' indicate the observable sequential NOE correlations and the arrows in 'b' indicate the magnetization transfer pathways used for obtaining the assignments. In 'b' the magnetization originates from the NH proton, travels to nitrogen and carbons as indicated and returns to NH for observation. The forward and backward transfers are indicated by different colours.

for structure calculations in all cases, the strategies for obtaining sequence specific assignments are different for small and large proteins^{5,7,9,38-41}. In small proteins where the number of signals is small, homonuclear ^1H - ^1H J and NOE correlated experiments proved sufficient. In larger proteins, however, extensive overlap of signals necessitated the use of heteronuclear and multidimensional NMR experiments. The two strategies are schematically indicated in Figs. 16a and 16b respectively. In Fig. 16a, the intraresidue connections are obtained from J-correlated experiments and the sequential walk through the polypeptide chain is performed via a set of NOEs. There are well defined NOE patterns for the regular secondary structures which help in obtaining the assignments. For example, in α helices, one observes a series of sequential NH-NH NOEs whereas in β sheets, one observes series of sequential NH_i - $\text{C}^\alpha\text{H}_{i-1}$ NOEs. Antiparallel β - sheets are also characterized by interstrand C^αH - C^αH NOEs. In Fig. 16b which represents the

strategy for large proteins, assignments of the ^1H and heteronuclei along the backbone are obtained first using a variety of triple resonance three dimensional experiments. The arrows in the figure indicate the pathways of magnetization transfers employed for obtaining sequential connections. This is then followed by other three dimensional heteronuclear experiments for assigning the side chains of the individual amino acids. In all these experiments the various magnetization transfer steps are essentially one-bond transfers and thus the assignments are independent of the three dimensional structures of the protein molecules.

After getting sequence specific assignment of the resonances by one of the procedures described above, several short and long range NOEs between the nuclei are identified and quantified using multidimensional NOE experiments. The NOE intensities are converted into approximate upper bounds on internuclear distances to be used as constraints during structure calculations. Further, three bond coupling constants are measured from J-correlated spectra and are converted into approximate torsion angle constraints. All these constitute the basic input for the structure calculation algorithms, Distance Geometry, and Simulated Annealing /Restrained Molecular Dynamics mentioned earlier. In both, the violations of the constraints are defined in terms of some sort of a potential and this potential is minimized using one of the different minimization algorithms. Finally in the refinement step, the NOE intensities are calculated for the convergent structures by relaxation matrix methods and these are matched with the experimental NOE intensities for acceptability of the structures. In the end one generates a family of 15-20 similar structures, all of which are supposed to represent the structure of the molecule. A detailed analysis of these structures in terms of pairwise *rmsds* along the length of the polypeptide backbone indicates, first of all the uniqueness of the structure and secondly the precision of the structure derived at different portions of the molecule. It is generally observed that the backbone in the interior of the protein is well characterized but the loop portions and the terminal regions are usually poorly characterized. Fig. 17 shows a NMR protein structure determined in recent years as an illustration^{42,43}. Protein structure determination by the above

strategies is labour intensive and analysis of spectra can take as much as 2-3 years for an average size protein of 150 amino-acid residues. Even so, at the present state of development of the techniques,

nearly 100 protein structures in different molecular size ranges are solved every year world wide.

The lack of precision of structure definition by NMR in any particular segment of a protein is a reflection of the local dynamism in the molecule. Segmental motions are known to occur in proteins and these result in average values for NOE intensities. A simplistic interpretation of the intensities in terms of the distances is then not appropriate and clearly no single distance is a satisfactory choice. Because of these, conservative distance estimates are given which leads to a greater variability in the structures. Additionally, dynamism in the molecule results also in poorer NOE intensities which in turn lead to fewer usable NOEs and greater errors in intensity measurements. These also contribute to the variability in the structures and hence to lower precision in the structure definition.

Protein structure and dynamism are influenced significantly by the environment. To investigate these effects it becomes necessary to compute the intrinsic conformational preferences and compare the structures with those derived from NMR under different experimental conditions. This will allow definition of the general principles and the various physical forces/interactions which govern the



Fig 17 Solution structure of Phospholipase A_2 protein molecule determined by NMR as an illustration (reproduced with permission from ref 42). Several equally probable structures are superimposed and the variations in these reflect the precision in the structure definition. The figure shows a backbone trace of the polypeptide chain.

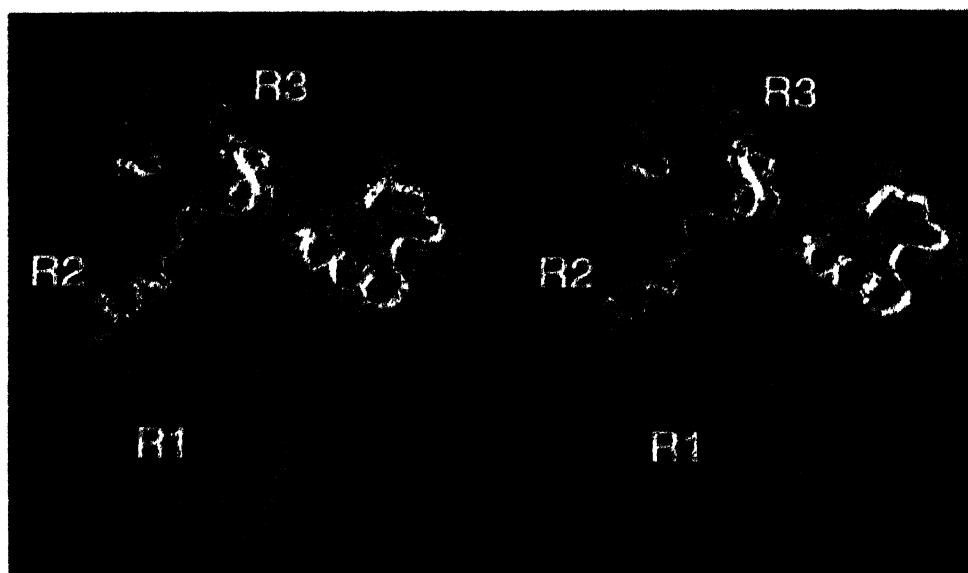


Fig.18 MD generated structure of the DNA binding domain of *Drosophila* Myb protein. The calculations were performed after immersing the protein in a box of water molecules and the conformational space was scanned by simulated annealing. The calculations used some constraints derived from secondary structure prediction algorithms and homology comparisons. R1, R2 and R3 are three domains in the molecule.

protein folding process. Molecular dynamics and simulated annealing are the best suited techniques for such investigations and many large molecules have thus been investigated. Calculations can be carried out in the presence and absence of water, to see the effects of solvation and they can be compared with the NMR derived results. Fig. 18 is a presentation of such an effort in which the theoretical 'solution' structure of a 160 residue DNA binding domain of *Drosophila* Myb protein investigated in our laboratory by simulated annealing and molecular dynamics calculations is shown. This structure differed quite significantly from the one derived when water molecules were not included in the calculations. Several tightly bound water molecules were detected and these

clearly influence the solution structures. There is no NMR data on this protein yet, but some of the general features may be compared with data available on smaller segments of the protein from other sources⁴⁴. The R1 domain showed formation of helices at the predicted positions in the absence of the constraints. The three segments R1, R2 and R3 do not have interactions between each other.

7 Protein - DNA Interactions

Recognition between nucleic acids and proteins lies at the heart of genetic expression and regulation inside the cell. Recognition of 'operator' and 'promoter' sequences by repressors, polymerases,

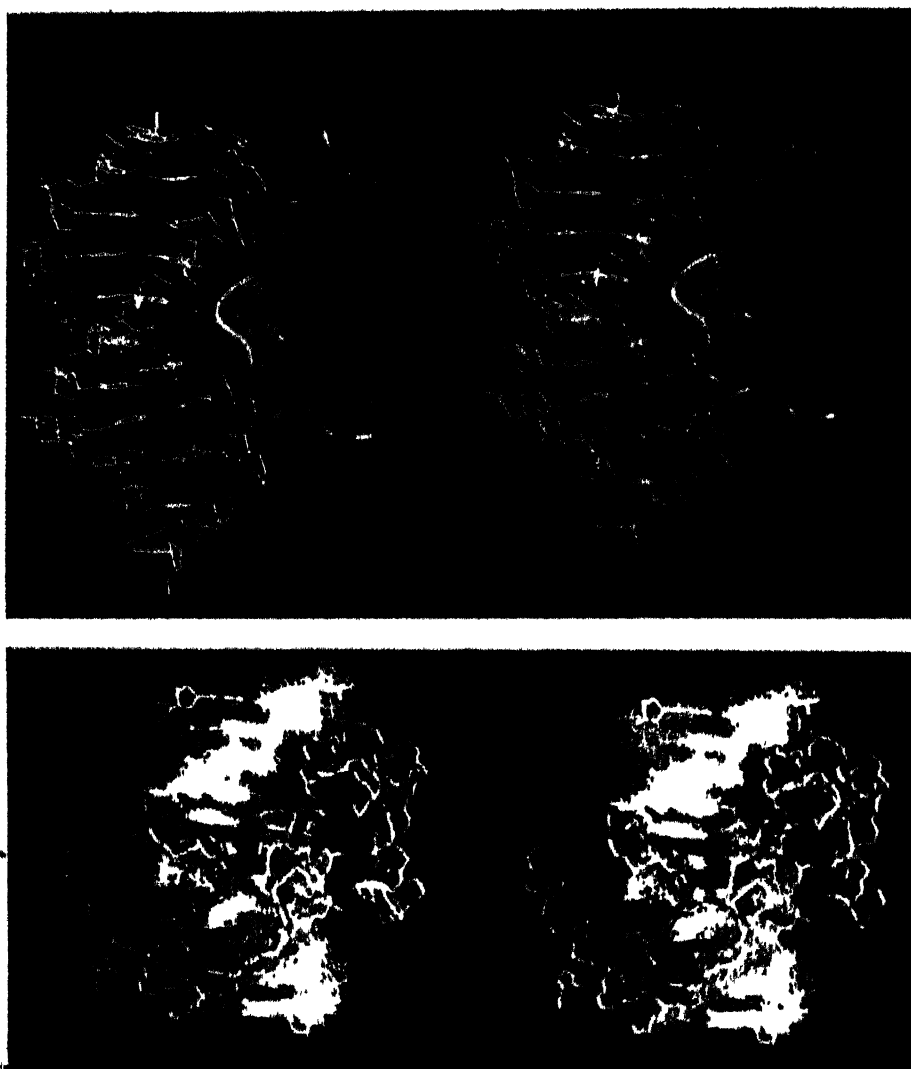


Fig 19 Solution structures of lac-DNA⁴⁶ (top) and MybR2R3 - DNA⁴⁷ (bottom) complexes determined to atomic resolution by a combination of various triple resonance techniques and using isotopically labelled protein and unlabelled DNA molecules (reproduced with permission). In the latter, a family of convergent structures is shown

transcription factors, cleavage sites by restriction enzymes are a few examples. Understanding these molecular interactions is a top priority in present day research all over the world, and NMR has been successful in unraveling the mechanisms of these interactions in several cases. Fig. 19 shows two recent examples of DNA-protein complex structures determined by heteronuclear NMR⁴⁵⁻⁴⁷. By using isotopically labelled proteins and unlabelled DNA molecules and by using special filtering techniques, it is possible to observe the NMR signals originating exclusively from either of the two molecules in a complex. NOE correlations between the protons on the protein and the protons on the DNA enable identification of contact points. Such a knowledge is a forerunner for the design of new and more efficient proteins.

Most of the DNA-protein complexes investigated so far concerned DNA duplexes. The structural investigations have indicated that the DNA binding proteins interact with DNA using one of a few well defined motifs, namely, 'helix-turn-helix', 'Zinc finger', 'leucine zipper' and 'helix-loop-helix'. The details of these have been reviewed extensively⁴⁸⁻⁵⁰. The examples shown in Fig. 19 make use of the helix-turn-helix motif. One of the helix sits in the major groove of the duplex DNA and the other helix orients itself in an orthogonal manner. Direct interactions occur between the sidechains of the recognition helix and the bases or the phosphates. The interactions with the bases are either H-bonding interactions or stacking of the aromatic residues over the bases. The phosphates are involved mostly in charge-charge electrostatic interactions but may also participate in H-bonding interactions. In addition to these, there will also be non specific interactions between the amino acid side chains and DNA backbone which contribute to the stability of the complex. It is observed in a few cases, lac repressor for example, that on complex formation the DNA and protein undergo partial conformational changes. In others, however, Myb DNA binding domain for example, no structural changes have been observed.

8 Future Directions

No one would have predicted twenty years ago that NMR would reach the heights it has reached today,

encompassing biology in the real sense of the term. Structural biology is as much an NMR branch of science as it is of X-ray crystallography. Structures of complex proteins, nucleic acids, protein-nucleic acid complexes, drug-nucleic acid complexes, protein-protein complexes etc. are being determined with breathtaking speed by both the methods. There have been instances where NMR structure has provided the starting model for X-ray structure determination and also *vice versa*. Clearly NMR has emerged as an alternative but complementary technique for macromolecular structure determination. The macromolecular concentrations used in NMR are comparable to the total protein concentration inside a living cell and the other conditions are adjustable to near physiological conditions.

Learning from past experience it would be clearly unwise and impossible to make a prediction far too long in the future as to the direction NMR research would take. The NMR methodology and technology are developing at a rapid pace with the objective of handling larger and larger molecules on the one hand - this includes complexes - and smaller concentrations of the molecules on the other, since larger molecules may not be soluble to the extent desired today and also because of the high cost of production of the labelled molecules. Assemblies of macromolecules could also be studied which provide insight into the cooperative structural changes, so crucial for a complex biological function.

During the last two decades, a large emphasis was laid on the determination of the average structures of the macromolecules in aqueous solutions. It is, however, known that the molecules are highly dynamic, exhibiting motions of different kinds. Different domains in a protein may have different motional characteristics and these would be important for adaptability of a protein molecule for a particular function. There are cases where the same molecule is responsible for a diverse set of functions. This can occur either by involvement of different domains in the molecule or in some other situations the same domain may undergo conformational changes. Thus there is increasingly greater interest today in defining the so-called dynamic structure of a molecule. In other words, efforts are being made to study the rates of

segmental motions, overall tumbling motions, anisotropy of motions, conformational exchanges, surface motions vs. motions in the interior of a protein, waters of hydration, ordering and trapping of water molecules in the interior of proteins, folding - unfolding equilibria in proteins, folding pathways and identification of intermediates, to name a few. Study of substrate - enzyme complexes would throw light on the mechanisms of enzyme actions.

As regards the biological systems, the primary consideration is their biological significance. While proteins continue to attract a lot of attention, RNAs have also emerged as hot favourites for many research groups because of the recent discoveries of ribozymes which are RNA catalysts, greater variety in RNAs, and greater involvement of RNAs in a number of biological functions. RNA and DNA aptamers and their complexes with the specific substrates are attracting substantial attention since these are expected to throw light on the intricate details of molecular recognitions.

The practice of NMR research, especially with reference to biomolecular NMR, has also changed dramatically. It has become highly multidisciplinary encompassing, biologists, spectroscopists,

chemists, computer engineers and physicists alike. Scientific collaboration is an absolute necessity today. In many labs, molecular biology research is an integral part of NMR activity. The production of desired materials by genetic engineering methods is expensive and the machine requirements have also increased quite substantially. It has become necessary to have quick and high access to sophisticated spectrometers for competitive research. In contrast to the situation in the eighties where most machines were run as service facilities, it is common now to see dedicated high field spectrometers for a small group of scientists. The spectrometer designs are improving rapidly, magnetic field strengths are spiraling upwards, and computer powers are pushing the sophistications and applications. Thus, to conclude, it remains anybody's guess as to what further heights NMR research would reach in the years to come.

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PHARMACEUTICAL APPLICATIONS OF NMR SPECTROSCOPY

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Early pharmaceutical applications of NMR spectroscopy were in the identification and characterization of chemically synthesized drugs or biologically active molecules derived from a variety of natural sources, which formed the basis of pharmaceutical products. However, several major advances in the technique have elevated the status of NMR spectroscopy as one of the most important tools in the area of rational drug design. Studies of drug-receptor interactions, more specifically protein-ligand interactions, provide the basic framework for understanding the mode of action of the drugs and in the design of more potent analogues. Immune system is a marvel of nature and immunosuppression by drugs or pathogens are topics of tremendous contemporary interest. Drugs that suppress the immune system are used to prevent the rejection of the organ transplant in major graft surgeries, while human immunodeficiency virus (HIV), the causative agent of the dreaded disease acquired immunodeficiency syndrome (AIDS), plays havoc with the immune system. Several breakthroughs have been made in these two areas of immunology in the recent past. NMR spectroscopy has provided the structure of immunosuppressant drugs, their receptors and the drug-receptor complex, providing a handle in deciphering the various steps involved in the immune response. The AIDS drugs inhibiting the enzyme, reverse transcriptase, which is involved in the transcription of the viral RNA to DNA, compete with the natural nucleosides, due mainly to the similarity of their molecular geometries and curtail the formation of viral DNA. HIV protease, a homodimer, is inhibited by small molecules. These drugs have transformed the therapy of AIDS. The structure obtained by NMR spectroscopy has thrown light on the role of flexibility of protease-inhibitor complex in the function of the protease. The development of a vaccine against AIDS is a challenging task due to high mutation rates of the virus. The peptides in main binding site of the HIV neutralizing antibodies have been investigated in detail, to obtain the immunogenic features. The recent applications of NMR in drug design using combinatorial chemistry have been very impressive. The presentation discusses the role of NMR spectroscopy in these specific pharmaceutical applications.

Key Words: NMR; Drugs; Immunosuppressant; AIDS; HIV; Immunophilins; Reverse Transcriptase Inhibitors; HIV Protease Inhibitors; Combinatorial Chemistry

Introduction

Chemical sciences have profoundly influenced the development of modern biology. Biological cofactors, metabolites, hormones etc. have been characterized and synthesized using the tools of organic chemistry. Similarly physical techniques like X-ray crystallography and NMR spectroscopy have been used to elucidate the structure of biomolecules. The impact of biology in chemistry however, can not be undermined. The complementary relationship between chemistry and biology is very well exemplified in the area of drug discovery where interactions involving the ligands and their receptors play a crucial role. Chemists and biologists have generated medicines of enormous importance to mankind. In spite of the increasing sophistication available to chemists, they are far from matching nature's efficiency in producing complex molecules that carry out the

intriguing process of life. Most of the leads of drug molecules have, therefore, come from natural biological sources like bacteria, fungi, plants and marine organisms. So much so, nearly all 'wonder drugs' in use presently have been derived from natural products. Further refinements and fine tuning is then done by organic chemists and medicinal chemists for effecting desirable changes for an efficacious and useful drug. To understand the biological activity of these molecules a knowledge of the three-dimensional structure of the drugs, receptors and the drug receptor complex is of great importance and NMR spectroscopy has a crucial role to play in such studies^{1,2}.

Historically applications of NMR in pharmaceutical sciences started in late 1950s. First applications were in assisting in the identification and characterization of chemically synthesized drugs or biologically active molecules derived from

a variety of natural sources. The role of NMR as essentially an analytical tool has now been supplemented by a more fundamental application in the design of new drugs, an outcome of major advances in NMR instrumentation and methodology in recent years. Recent publications of several books, monographs and reviews amply demonstrate the importance of NMR in drug design³⁻⁷.

Real pharmaceutical applications of NMR spectroscopy started in 1960s and 1970s, with the determination of structure and conformation of biologically important molecules with molecular masses upto about 1 kDa. Conformational information is obtained from the chemical shifts (δ), indirect spin-spin couplings (J) and nuclear Overhauser enhancements (nOe). The increasing availability of higher field spectrometers and sophistication in instrumentation enhanced several fold the complexity of molecules amenable to NMR study after mid 1970s. The development of two dimensional NMR (2D NMR)¹, a big-leap in NMR methodology, has enabled a wide range of new applications in the area of drug design. These applications have mainly exploited the usefulness of biologically active peptides and peptidomimetics as potential targets for analogue based drug design and proteins in receptor based drug design. The advent of 3D and 4D NMR^{8,9} coupled with very high field NMR spectrometers (^1H frequency of upto 800 MHz) has extended the size of biomolecules that can be studied at molecular level to about 40 kDa, though recently a homodimer of *Escherichia coli* chemotaxis kinase, having a molecular weight of 142 kDa, has been studied¹⁰. The ease of uniformly labelling the proteins with ^{13}C and ^{15}N has also contributed very significantly in these advancements.

NMR spectroscopy is being used very widely in the area of drug design. Such studies usually aim at obtaining: a) the structure of the drug molecule, its receptor bound conformation as well as its dynamics at the receptor site, b) the structure of the receptor and its complex with the drug, which provides the information on the binding site and functional group interaction participating in the ligand recognition and binding.

Immunosuppressants

Importance of some of the aspects mentioned above is very well demonstrated in the two

problems of tremendous interest in contemporary immunology. In this article, we discuss some specific structural studies involving immunosuppressant drugs and drugs for the treatment of acquired immunodeficiency syndrome (AIDS) in detail. Most of the discussion that follows pertains to NMR data, additionally supported by molecular dynamics calculations^{11,12} and X-ray diffraction studies.

The immune system is a marvel of nature. It is not controlled by any central organ and functions through an exotic information network which is constantly fighting and engaged in imperceptible war against foreign invaders, like bacteria, viruses, fungi and parasites. Already a large body of information exists about the immune system and more and more fascinating details are becoming available. Immunosuppressants, used for preventing the rejection of the transplanted organs in surgery, have played an important role in our understanding of the immune system. Tremendous efforts in finding a cure for AIDS, the dreaded disease, caused by infection with human immunodeficiency virus (HIV) are being made by scientists all over the world for several years. Understanding the finer details of the functioning of the immune system may help in the prevention and cure of AIDS.

Immunosuppression has enormous medical relevance. The success of organ transplantation surgery is based to a large extent on the miracle drug cyclosporin A (CsA)¹³ (Fig. 1A) obtained from fungal sources. With the use of CsA we can traverse the fine line between the graft rejection and infection of the patients due to a necessary suppression of the immune system. CsA binds with high affinity (dissociation constant, $K_d=30$ nM) to an abundant cellular protein (≈ 5 nM, upto 1-2% of the total cellular protein), called cyclophilin (CyP)¹⁴. Subsequently another drug, FK506¹⁵ (Fig. 1B), which is about 10 times more active than CsA, was discovered. FK506 binds to another ubiquitous protein, referred to as FK binding protein (FKBP)^{16,17}, with very high affinity ($K_d=0.4$ nM). Rapamycin¹⁸ (Fig. 1C), which is structurally very similar to FK506, is another immunosuppressant compound (not yet approved as a drug) with even larger activity than FK506 and binds with greater affinity ($K_d=0.2$ nM) to FKBP. Both cyclophilin and FKBP are enzymes (referred to as rotamases) with peptidyl-prolyl *cis-trans*

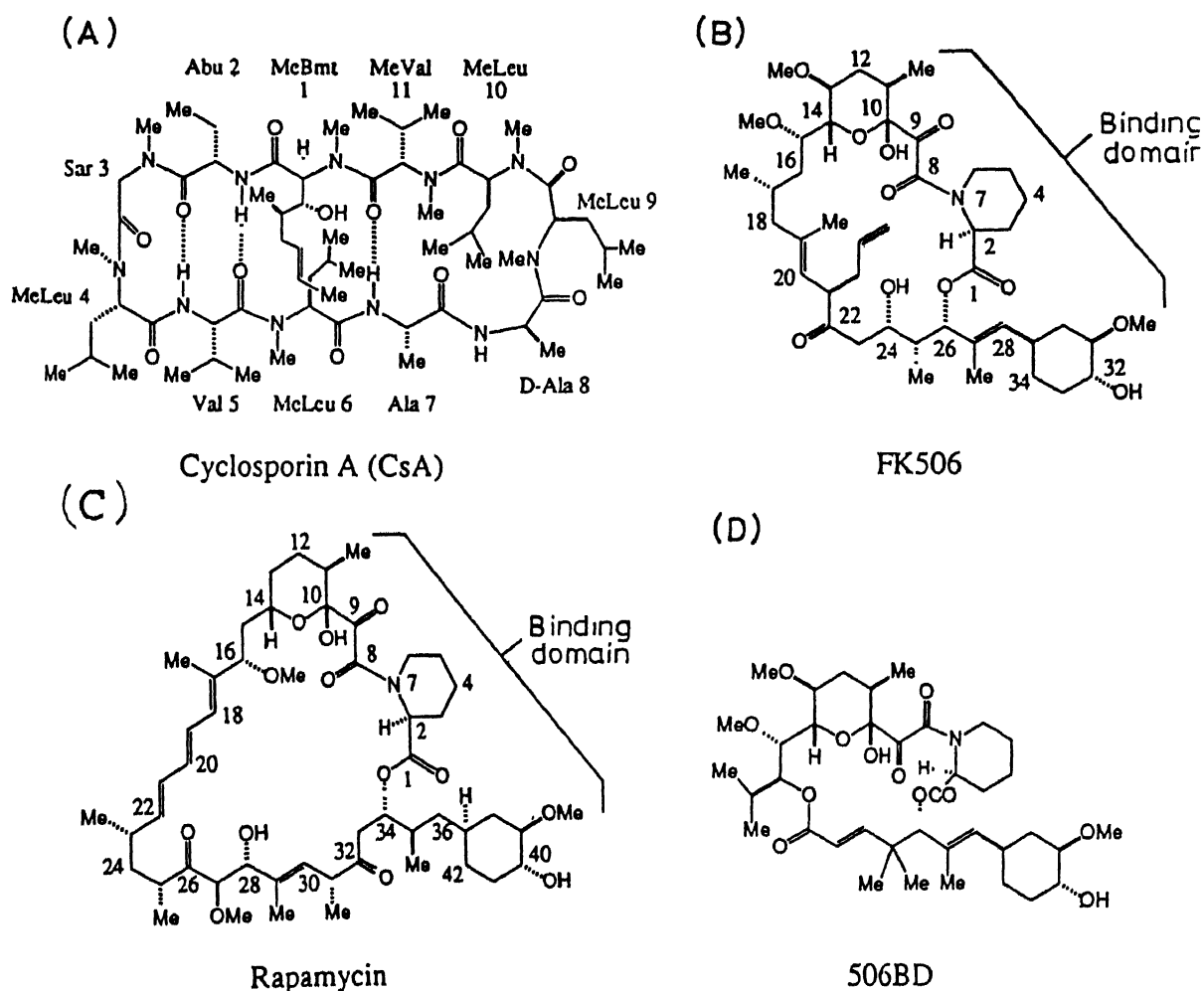


Fig 1 Chemical formula of (A) cyclosporin A (CsA), (B) FK506 (C) rapamycin (D) 506BD Binding domain of FK506 and rapamycin is shown The region opposite to the binding domain is the effector domain

isomerase activity^{14,16,17}. The enzymatic activity of CyP is inhibited by CsA (inhibition constant, $K_i=6$ nM)¹⁴ and that of FKBP by FK506 ($K_i=0.4$ nM)^{14,15}. These enzymes collectively called immunophilins speed up the isomerization of peptide-proline bonds in polypeptides and thereby facilitate protein folding. Immunophilins may be thus helping to refold proteins that might have changed their conformation during transport through membranes¹⁹⁻²¹. Importantly the binding of these active molecules blocked the isomerase activity of immunophilins, thus inhibiting their normal cellular functions. The availability of these drugs has led to a better understanding at the molecular level of the biochemical signal transduction involved in the immune response. All these molecules inhibit cell proliferation, but exert their actions at different stages of T cells activation. CsA and FK506 inhibit Ca^{2+} dependent

signalling pathways, which result in the prevention of transcription of several genes, including those encoding for interleukin-2 (IL-2), which are normally activated by stimulation of T cell receptors (TCR). Rapamycin, on the other hand, intervenes in the Ca^{2+} independent pathway, interfering in events more closely related to DNA synthesis, by inhibiting the differentiation and proliferation of T cell, normally mediated by the IL-2 receptor (IL-2R)²².

The similarity in biological functions, coupled with the inhibition of two different rotamases by these drugs, resulted in a belief that the immunosuppression is due to the inhibition of rotamase. Strong evidence against the "rotamase hypothesis" came from rapamycin and another structurally similar molecule 506BD²³ (Fig. 1D), a synthetic ligand which binds to FKBP with high affinity ($K_d=20$ nM). Due to their structural

similarity with FK506, rapamycin and 506BD bind tightly to FKBP and inhibit the rotamase activity of the enzyme ($K_i=0.2$ nM for rapamycin and 5 nM for 506BD)^{23,24}. However, while other drugs lead to immunosuppression, 506BD does not, implying that inhibition of rotamases is not enough for a molecule to be immunosuppressant.

It was subsequently shown that calcineurin (CN), also called protein phosphatase 2B, a calmodulin dependent serine/threonine protein phosphatase²⁵, possesses all the predicted biochemical properties of a target for both CyP-CsA and FKBP-FK506 complexes and gets specifically inhibited by them²⁶. CN is a heterodimeric protein composed of two subunits calcineurin A (CNA), which contains the calmodulin-binding and phosphatase active sites and calcineurin B (CNB), which is a Ca^{2+} binding protein. Calmodulin binding to CNA enhances the phosphatase activity of the enzyme by about ten times. The picture emerging from a series of elegant experiments shows that, when FKBP-FK506 and CyP-CsA encounter the target protein CN, the exposed domain of the complex binds to it. Thus in the pentapartite complex, drug-immunophilin-CNA-CNB-calmodulin, CsA and FK506 behave like molecular glue. Structural dissimilarities of CsA and FK506 as well as the unrelated sequences of CyP and FKBP make these results all the more remarkable. To understand the basis of molecular recognition within these pentameric complexes some detailed structural analyses have been undertaken and lot more experiments are still required to be performed. The question pertaining to the role of the complex has been addressed later. A series of elegant NMR experiments have provided information on the ligand receptor interaction which are additionally supported by X-ray diffraction and molecular dynamics (MD) calculations. The discussions on the structure in this paper represent overall view from these techniques.

Structure of Cyclosporin A, Cyclophilin and their Complex

Cyclosporin A (Fig. 1A) is a neutral cyclic undecapeptide consisting of several unnatural amino acids, seven of them N-methylated. All the amino acids are lipophilic, making it water insoluble. A large number of synthetic and natural analogues have been used for structure-activity

relationships. Kessler *et al.* have performed elegant NMR studies on CsA²⁷⁻³⁴. In solvents of low-polarity like CDCl_3 , C_6D_6 and THF-d_8 ^{27-31,34}, CsA takes very similar conformations. On the other hand, in solvents of higher polarity, the changes in the conformations are directly visible in the NMR spectra; many conformations exist in equilibrium inter-converting slowly on the NMR time scale. In DMSO, at least seven conformations can be observed³⁰. Because CsA is insoluble in water, as a first step in understanding the function and interactions of CsA with CyP, detailed structural studies have been undertaken in CDCl_3 . The first such study of cyclosporin²⁷ could not clear some ambiguities and subsequently some very high quality spectra were obtained at 600 MHz³³. Using 117 distance constraints, structure refinement and modelling studies revealed a structure shown in Fig. 2 as a stereo plot. The structure is very similar to that obtained from X-ray diffraction²⁹ with some of the notable features as:

- 1 The amide bond between MeLeu 9 and MeLeu 10 is *cis*.
- 2 All four NHs are involved in internal H-bonds, with Val 5 NH and Ala 7 NH partially participating in three center H-bonds (H-bonds are very similar to that obtained from crystal structure). The MeBmt(4(R)-4-[(1*E*)-but-2-enyl]-4,N-dimethyl-L-threonine) side chain is folded over the backbone, like the crystal structure, as against the earlier NMR observation, where extended structure was obtained and MeBmt 1 OH is involved in H-bond with the MeBmt 1 CO.
- 3 Existence of the type II' β turn involving Abu 2-Sar 3-MeLeu 4-Val 5 was definitely confirmed, unlike earlier study where such details could not be obtained.
- 4 The residues MeVal 11-MeBmt 1-Abu 2-Sar 3-MeLeu 4-Val 5-MeLeu 6-Ala 7 form a β sheet structure.
- 5 Six of the seven N-Me point outside the ring and thus expose to the solvent. Only MeVal 11 is directed towards the centre of the macrocycle.
- 6 The residues 7-11 form an irregular structure stabilized by hydrogen bond between D-Ala 8 NH-MeLeu 6 CO and several van der Waals interactions.

Several studies on the structure of CyP have been undertaken in aqueous solutions³⁵⁻³⁸ CyP

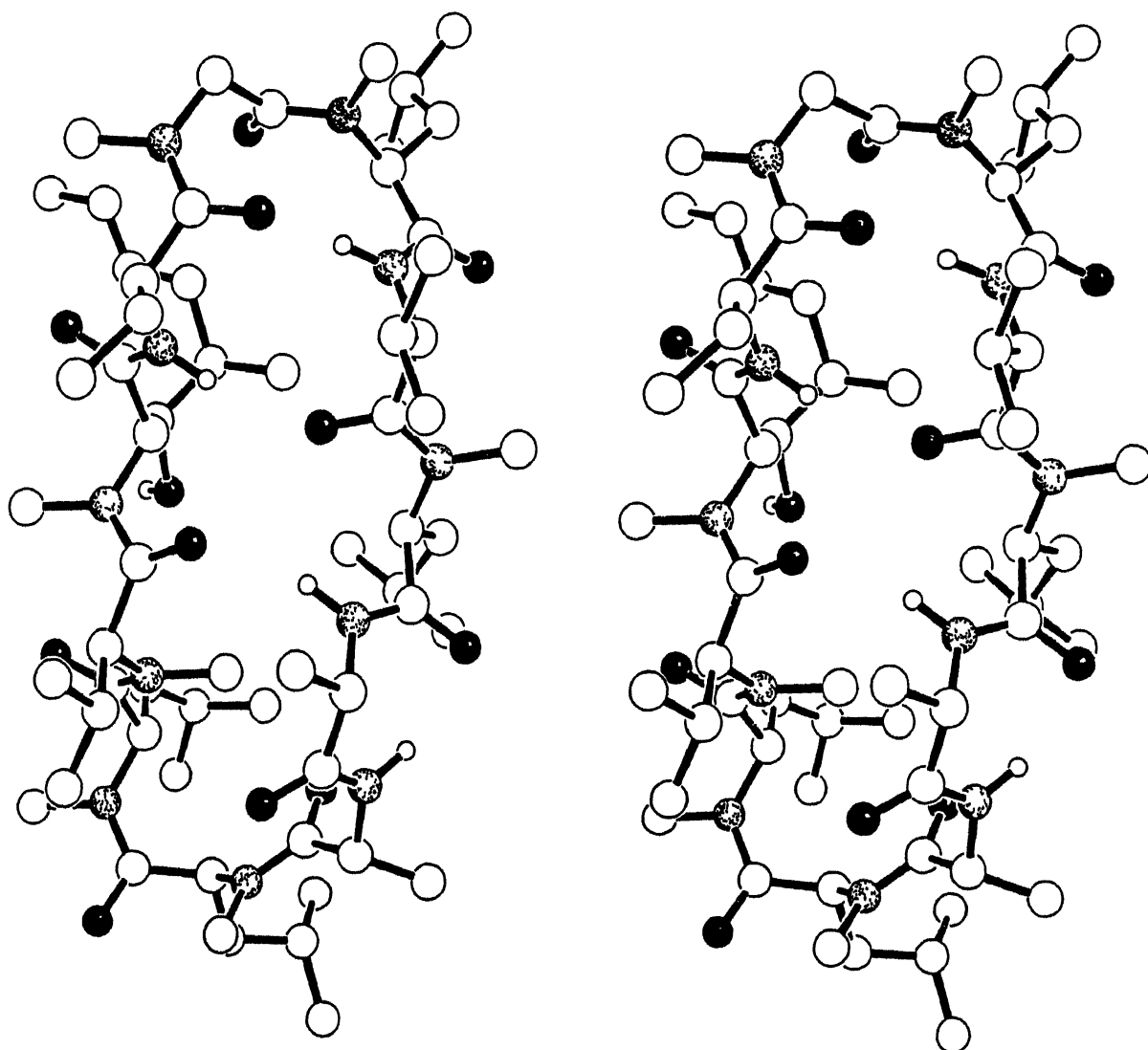


Fig 2 Stereoplot of CsA structure obtained by restrained molecular dynamics calculations. The type II' β turn between Abu 2 and Val 5 is at the top of the molecule. The O-atoms are filled and the N atoms are striped (Adapted with premission from H Kessler *et al* *Helv Chim Acta* 73 (1990) 1669 Copyright ©1990 by the Neuc Schweizerische Chemische Gesellschaft

consists of single polypeptide chain with 165 amino acids having a molecular mass of 17.8 kDa. It is a roughly spherical molecule with a radius of about 17Å. CyP has two α helices and eight stranded β -barrel that has a +1,-3,-1,-2,-2,-3 topology (Fig. 3B). The barrel consists of two roughly perpendicular four stranded β sheets connected by short junctions at residues Leu 98-Gly 13 and Phe 53-Ile 156. Inside the barrel, a tightly packed core contains most of the hydrophobic side chains. Other hydrophobic residues are located in the contact region of the two amphipathic helices with the β -barrel and in the CsA binding site (to be discussed later).

There is a structural similarity between CyP and the super family of β -proteins family³⁹ involved in ligand transport including retinol binding protein (RBP)⁴⁰, bilin binding protein⁴¹, insecticyanin⁴² and β -lactoglobulin⁴³. Most of these molecules encapsulate their ligands in the β barrel core. By contrast the barrel with hydrophobic residues and the ligand binding site in CyP is outside the barrel. The topology of cyclophilin also differs from the simple (+1)_n up-and-down fold in RBP class of proteins or the (-3,+1,+1) Greek key topology that is most frequently found in antiparallel β -barrel proteins. The two cross over connections Gly 64-Ile 97 and Thr 116-Gly 130 (Fig. 3C), in particular

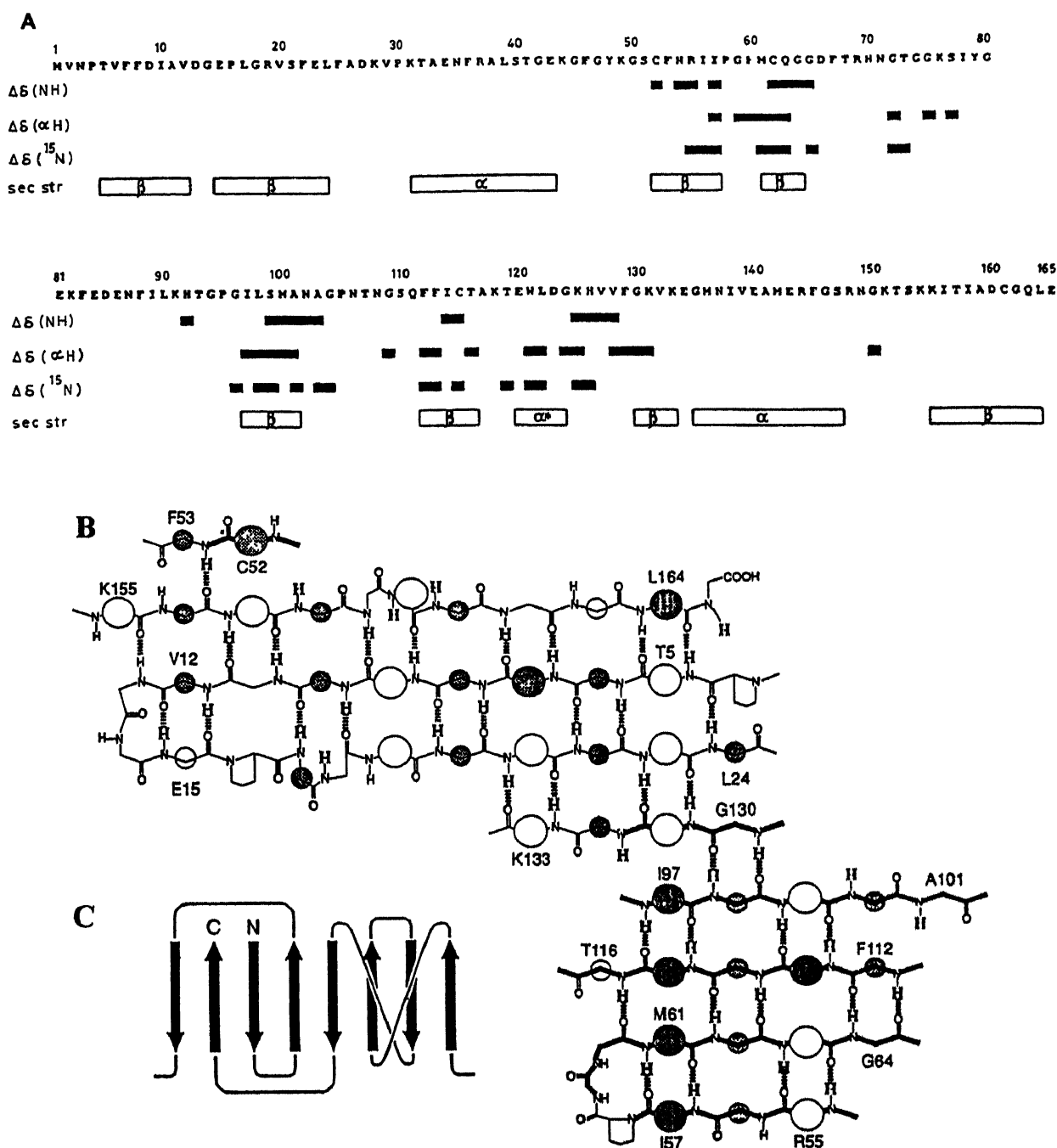


Fig 3 Summary of NMR results with CsA binding site and the secondary structure and topology of CyP (A) Amino acid sequence, chemical shift data used for delineating CsA binding site and the regular α -helical and β -sheet structures (α^* depicts a short α helix or a turn like structure) The residues displaying significant backbone chemical shift variation on binding of CsA are shown with black bars. (B) Schematic representation of the antiparallel β -sheet in CyP showing a global (+1,-3,-1,+1,-2,-3) topology of the β -sheet. (C) Drawing showing the sequence positions of the individual β -strands, the hydrogen bonds, the distribution of the hydrophobic side chains and the location of residues with large chemical shift changes on binding to CsA. The presence of hydrogen bonds is identified with dashed lines. The amino acid side chains are represented with circles at C_α carbon positions using the following notation: big circles, side chains toward the reader, small circles, side chains pointing away from the reader, shaded circles, hydrophobic residues; empty circles, polar and charged residues. For Pro a five-membered ring is drawn. Ala, Gly, Tyr and His have no symbol at the C_α position. Essentially all side chains below the plane of the β sheet are hydrophobic (adapted with permission from J Kallen *et al* *Nature* **353** (1991) 276 Copyright © 1991 by the Macmillan Magazines Ltd)

represent an unusual topological feature. As both loops lie on the outside of the barrel, the (-2,+1,-2) topology requires that the two loops cross each other. The unusual left handed connection of the Thr 116-Gly 130 loop may be rationalized by the length of the loop which may accommodate a variety of local conformations. Fig. 4, shows a ribbon diagram of CyP clearly depicting the secondary and tertiary structure.

The study of CyP-CsA complex in solution shows very small changes in the structure of CyP on complexation with CsA⁴⁴. The CyP-CsA complex structure is also similar to that observed in the solid state for CyP⁴⁵ and CyP-tetrapeptide complex³⁵. The structure of the complex is stabilized by several hydrophobic interactions. Fig. 3A depicts the residues which show significant chemical shift variation on binding to CsA. Chemical shift differences are indicated by black bars when they exceed the limits $\Delta\delta(\text{NH}) = 0.1$ ppm $\Delta\delta(\alpha\text{H}) = 0.05$ ppm and $\Delta\delta(^{15}\text{N}) = 0.5$ ppm. The side chains of CyP residues Trp 121, Phe 60,

Ile 57, Leu 122, Phe 113, His 126, Ala101, Ala 103 and Thr 73 form a hydrophobic pocket that interacts with the hydrophobic surface of the CsA residues 9-11 and 1-3. A large number of intermolecular hydrogen bonds also stabilize the structure of the complex. The NMR data show hydrogen bonds between Arg 55(ηNH)-MeLeu 10(CO), Trp 121(ϵNH)-Leu 9(CO), and Asn 102(CO)-Abu 2 NH. The C α -C' bond of MeVal 11 appears to mimic a twisted amide bond, which is proposed as a mechanism of the rotamase catalysis of CyP and will be discussed in greater detail later.

The structure of CsA in the complex was revealing⁴⁶. This information has been obtained by various groups⁴⁷⁻⁴⁹. The earlier studies used isotope labelling^{47,48}, to suppress the spectral lines due to CyP, by either filtering them by heteronuclear editing⁵⁰ or uniform ^2H labelling of the CyP⁴⁹. These manipulations provided information only on the bound CsA which of course agreed with complete structure of CsA-CyP complex obtained subsequently⁴⁶. The isotope labelling followed by



Fig. 4 A ribbon diagram of cyclophilin (CyP) (adapted with permission from K Wuthrich *et al* *J Mol Biol* 272 (1997) 64 Copyright © (1997) Academic Press Limited)

heteronuclear editing experiments demonstrate the power of NMR spectroscopy for drug-receptor studies. In an unlabelled system the large number of protons from the receptor protein would interfere with the observation of the resonance lines of the ligand. Binary complexes are ideally suited for studies with efficient labelling schemes, as the two components can be labelled separately with ^{13}C or ^{15}N before complex formation. Suitably designed sequences can be used for heteronuclear editing to separate the lines from the two components of the complex. Complete deuteration of one of the molecules in the complex enables the editing in a rather obvious and simple manner. Such studies represent an attractive avenue for the use of NMR and related fields on drug design. A very important finding emerged that showed the

structure of CsA is strikingly different from that in CDCl_3 solution as well as the one obtained in the crystalline state by X-ray diffraction. The salient features of the structure shown in Fig. 5 are.

- 1 The bound CsA lacks regular secondary structure; all amide bonds taking a *trans* geometry.
- 2 It has no intramolecular H-bonds.
- 3 But for MeLeu 9 and MeVal 11, rest of the five NMe groups are directed towards the centre of the macrocycle enabling them to make large number of van der Waals contacts. This implies that compared to the free CsA, the bound CsA structure has essentially been turned inside out.
- 4 Ten of the C=O and four of the NH are either

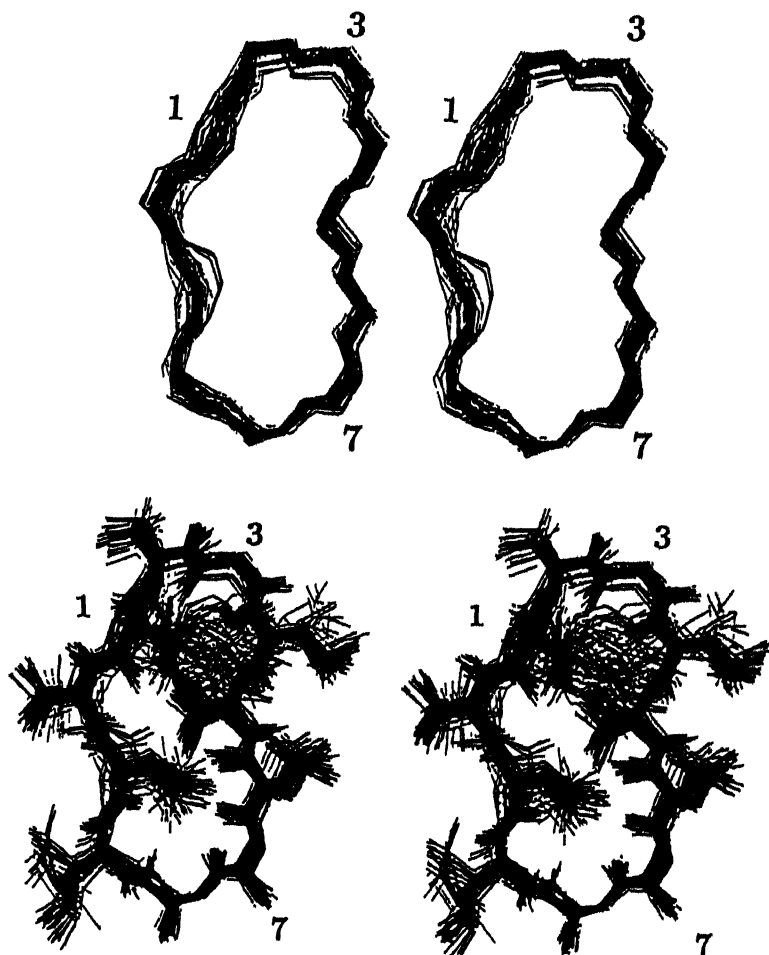


Fig. 5 Structure of CsA bound to CyP stereoviews are shown of a superposition of several best energy minimized structures. (A) The bonds connecting the backbone atoms (B) the bonds connecting all heavy atoms with the same orientation as in (A) (adapted with permission from K. Wuthrich *et al. Biochemistry* 30 (1991) 6563 Copyright © American Chemical Society).

partially or fully exposed enabling them to interact with CyP

As already mentioned residues 9-3 show many internuclear contacts with CyP and constitute the binding domain, while residues 4-8 do not interact with CyP. The residues 4-8 possibly bind with CN when CsA is bound to CyP and should correspond to effector domain of the CsA. It is interesting to note that CsA backbone from Sar 3 NH to Ala 7 CO, with the exception of MeLeu 4 resembles one strand of pleated β sheet. It is suggestive that the interaction between CN and CyP-CsA may be similar to those between strands of a β sheet. Residues 3-7 present a regular surface to CN and may make several hydrophobic contacts with it.

Recent work by Wuthrich *et al.*³⁸ on CyP is an elegant study which has in addition reviewed the three dimensional structures of CyP and CyP-CsA complex (Fig. 4). Detailed comparison of free CyP revealed subtle but significant conformational differences that can be related to lattice constants in the crystal structures in most cases. ¹⁵N relaxation times and NMR line shapes analyses for CyP in the free form and Cyp-CsA complex revealed transitions of polypeptide loops surrounding the ligand-binding site from locally flexible conformations in the free protein, some of which includes well defined conformational equilibria, to well defined spatial arrangements in the CyP-CsA complex. The NMR structure presents a highly relevant reference for studies of changes in structure and internal mobility of the binding pocket upon ligand binding, and possible consequences of this conformational variability for CN recognition by the CyP-CsA complex.

NMR results on the structure of the CyP-CsA complex are consistent with the previously observed structure-activity relation of modified CsA analogues. For example, the marked decrease in CyP affinity for CsA analogues substituted with larger side chains at the residue 11 can be rationalized by the tight fit of the MeVal 11 in the middle of the binding pocket^{51,52}. In contrast MeLeu 9 and Abu 2 are located near the sides of the binding pocket and a variety of other substituted amino acids can be accommodated without substantially influencing the affinity to CyP. Similarly since the residues 5 to 8 do not interact with CyP, and as expected, can tolerate the modifications. Some analogues, like (MeAla 6) CsA and (D-Abu) CsA, with modifications at these

sites, despite binding tightly to CyP are inactive. This is consistent with their decreased affinity to CN. Though the pentapartite complex, CsA-CyP-CNA-CNB-Calmodulin, is too big to study by NMR, some details are available from X-ray diffraction in crystalline state⁵³.

CsA has several undesirable effects arising due to CsA mediated inhibition of CN in cells, outside the immune system. Recently Schreiber *et al.*⁵⁴ have synthesized a modified CsA (CsA*) that does not bind to CyP and consequently does not inhibit CN in cell. They also modified the CyP (CyP*) in its CsA binding pocket which promoted high affinity complexation with the CsA* ($K_d = 9$ nM). The modified drug-receptor complex (CyP*-CsA*) inhibited CN ($IC_{50} = 25$ nM). The ability to inhibit CN in a tissue specific fashion, for example, through the expression of CyP*, may provide a method to understand the role of CN in different cell types. These studies have raised some interesting questions and may play a role in development of new immunosuppressant.

Structure of FK, Rapamycin, FKBP and FKBP-Ligand Complex

NMR studies have revealed that the structure of free FKBP is characterized by a five stranded antiparallel concave sheet with a novel +3,+1,-3,+1 loop topology^{55,56}. The strands of sheet correspond

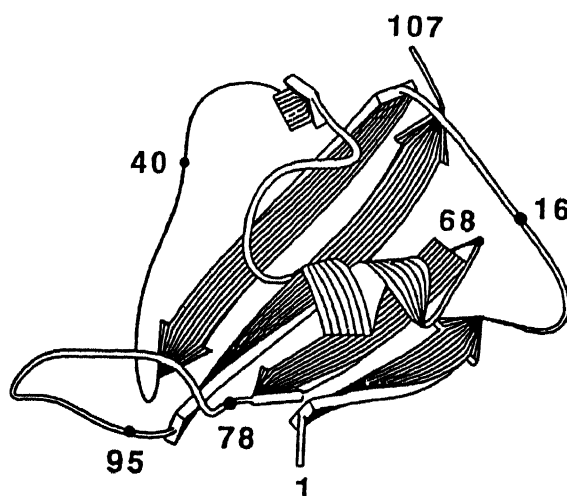


Fig 6 Carbon ribbon diagram of human FKBP. The five stranded sheet, an α -helix and the connecting loops are indicated. Also shows the N- & C-termini and other residues in the protein are also shown (adapted with permission, from S W Michnick *et al* *Science* 252 (1991) 836 Copyright © 1991 by the American Association for the Advancement of Science).

to residues 2-8, 21-30, 35-38 with 46-49 (interrupted by a loop at residues 39-45), 71-76 and 97-106 and run almost at 90° to the long molecular axis (Fig. 6). The only helix, containing residues 57-63, is amphipathic and runs along the long axis of the enzyme and lies against the sheet, forming a tightly packed hydrophobic core. The core consists of entirely aliphatic and aromatic residues. The conserved aromatic and aliphatic side chains of Tyr 26, Phe 36, Phe 46, Val 55, Ile 56, Trp 59, Tyr 82 and Phe 99 line a shallow cleft at the N terminus of α helix, forming the FK506 and rapamycin binding sites^{57,58}. The side chains of these residues are well defined in both the free and complexed (with ligand) FKBP. Both FK506 and rapamycin fit into the binding pocket tightly and have a number of similar hydrophobic and hydrogen-bonding interactions with the protein. The FKBP binding domain of FK506 extends from the C24 hydroxyl group through the diketo pipicolinyl moiety and the pyranose ring to the C15 methoxy group. This finding was anticipated on the basis of structural analysis of FK506 and rapamycin and resulted in the design of 506BD and other interesting analogues⁵⁹. The unsubstituted cyclohexyl ring (C26-C34) and the carbon atoms C18-C23 of FK506 do not make contact with FKBP and remain exposed to the solvent. This solvent exposed portion is referred to as the effector domain and should bind with calmodulin-CN to form a pentapartite complex. For rapamycin the orientation of the cyclohexyl ring (C34-C42) is different from that in FK506 and makes several contacts to FKBP. From extremely high field shifts of pipicolinyl methylenes and several NOE crosspeaks, it was concluded that pipicolinyl moiety makes van der Waals contact with the indole ring of Trp 59, at the back of the binding pocket, and with the side chains of Tyr 26, Phe 46, Val 55, Ile 56 and Phe 99 at the sides of the pocket. The pyranose ring comes in close proximity to residues Tyr 26, Asp 37, Tyr 82, His 87 and Ile 90. The C24-C26 region of FK506 and C28-C32 of rapamycin also makes close contact with Phe 46 and Glu 54. So many complimentary hydrophobic contacts support the large binding constant of FK506 and rapamycin with FKBP.

In addition to large number of van der Waals interactions, several intermolecular hydrogen bonds are formed between the ligands and FKBP. For both ligands, the hydrogen bonds between the

C1 ester carbonyl-Ile 56 NH, the C'8 amide carboxyl-Tyr 82 phenolic OH, C10 hemiketal OH-Asp 37 carboxylate are observed. Glu 54 CO makes hydrogen bonds with C24 hydroxyl of FK506 and C28 hydroxyl of rapamycin. An additional hydrogen bond between C40 hydroxyl-Glu 53 side chain CO is observed for rapamycin, but does not exist in case of FK506. The hydrogen bonds involving Ile 56 NH, Glu 54 CO and Glu 53 CO resemble antiparallel β -sheet-like contacts, often seen between protein and peptide ligands. These hydrogen bonds then extend the portions of FK506 and rapamycin, which can be treated as peptidomimetic, to include the regions between C1 ester carbonyl group and C24 (C28 for rapamycin) hydroxyl group⁷.

Since the inhibition of phosphatase activity of the CN takes place only with the binding to FKBP-ligand complex, it is important to analyze the differences between the free and bound form of both the protein and ligands. Majority of residues in FKBP-ligand complex are not appreciably perturbed by ligand binding. Even the aliphatic and aromatic residues making direct contact to ligand (Tyr 26, Phe 46, Val 55, Ile 56, Trp 59 and Phe 99) show only minor conformational changes between the bound and free protein. The regular structures involving sheets and α helix also show very small variations in the two structures. However, there are several loops in FKBP, located at the rim of the binding pocket, that seem to show significant changes in their structure and dynamics on ligand binding. For free FKBP the seven residue bulge, Ser 39 to Pro 45, appears to be disordered, while in FKBP-FK506 this region shows large variations of chemical shifts, implying that many of the residues in this region form hydrogen bonds resulting in change in conformation and mobility of the bulge. Interestingly on ligand binding the region Lys 34 to Lys 44 is the most charged, suggesting that it plays an important role in binding to the CN.

Significant changes are also observed on ligand binding in the sequence Tyr 82 to His 94. This loop in free FKBP is again poorly defined and the region appears to have at least two distinct conformations that inter-convert slowly on the NMR time scale. On ligand binding, this loop shows a distinct well defined conformation, stabilized by several hydrogen bonds within the protein and has two type II β turns involving residues 87-90 and 92-95 and van der Waals

contacts between the ligand and enzyme. These changes again are suggestive of the fact that residues Tyr 82 to His 94 may be involved in the CN recognition along with the residues Ser 39 to Pro 45. This would then result in a CN binding site that spans almost 20 Å and contain regions of high hydrophobicity from the effector domains of the ligands and high positive charge density from the protein. To find a definitive answer more detailed experiments would be required.

Though the differences in the protein structure on ligand binding are small, the ligand itself shows

major structural changes. In organic solvents, NMR studies show that FK506 exists as a 2:1 mixture of *cis-trans* amide bond rotamers^{15,60-62}. NOE restrained molecular dynamics studies have revealed structure of both the rotamers^{61,62} (Fig. 7). These structures differ considerably from those in the solid state¹⁵. The *cis* structure, in the binding domain is very similar to the structure in the crystalline phase. However, there are large differences in the torsional angles in the effector domain, involving the C-C bonds of C20 to C27 chain. This produces an extended and relatively flat

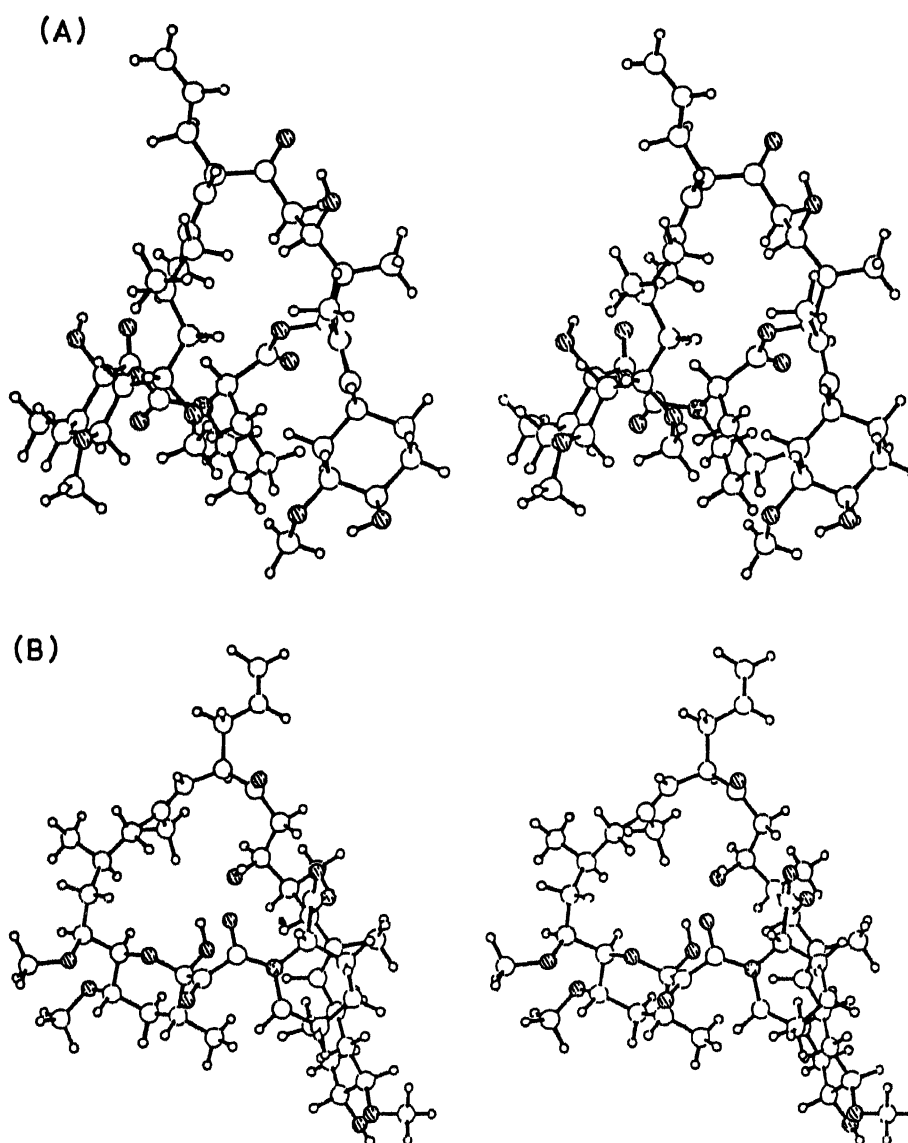


Fig. 7 Stereostructure of low-energy conformations of FK506 from restrained molecular dynamics (A) conformation containing a *cis* peptide bond between atoms 7 and 8 (B) conformations with a *trans* peptide bond between atoms 7 and 8 (adapted with permission) from P Karuso *et al J Amer Chem Soc* 112 (1990) 9434 Copyright © 1990 by the American Chemical Society).

structure and cyclohexyl residue extends away from the macrocycle in the solid state. In solution it is wrapped under the macrocycle due to C25-C26-C27-C28 dihedral angle of 91° . Interestingly the *trans* isomer in solution has a different structure than that in the solid state at the FKBP binding domain, but the rest of the molecule is almost similar. The differences in the torsional angles at C1-C2 and C9-C10 accommodate the change from *cis* to *trans* bond. Thus only other major changes exist around C16 to C19.

The structure of bound FK506 is drastically different from that of the free ligand (both in the solution and solid state⁶³). The major change in solid state is the *trans* amide bond in the bound ligand while it is *cis* (the major component) in solution. Large changes are observed in the torsional angles around C1-C2, C14-C15, C15-C16 and C16-C17 bonds in solid as well in solution state. These changes result in a more compact shape for the binding domain and forms a hydrophobic core of the ligand.

As against FK506, the free and bound conformations of rapamycin are very similar^{7,64}. Rapamycin has a *trans* amide bond in crystalline state, while in organic solvents it exists as a 4:1 mixture of *trans* and *cis* amide bond rotamers. It also has the pyranose moiety folded under the macrocycle and cyclohexyl moiety extended outward from the body of the molecules. In fact, the conformation of the FKBP binding domain of

rapamycin is almost identical to that of FK506. The implicit greater preorganization of rapamycin might be thought to provide greatly enhanced binding relative to FK506, which in practice has a binding constant only twice as large.

The structural studies of FKBP-FK506 attempted to provide a model for the rotamase inhibition by ligands⁶⁰. One mechanism suggested was the formation of tetrahedral enzyme substrate adduct. The rotation about the C-N bond in the adduct followed by the expulsion of the enzyme results in an amide bond isomerization⁶⁵. The second mechanism suggested the binding of a transition state structure, which contains a twisted or distorted amide bond⁶⁶ (Fig. 8). The energy for isomerization in this model becomes available due to favourable noncovalent interactions between the enzyme and a peptide substrate.

The mechanism involving the tetrahedral intermediate was ruled out from some elegant NMR experiments which showed the non-existence of tetrahedral adduct expected due to nucleophilic attack of a side chain of the enzyme on one of the electrophilic carbonyl in FK506 at C8 or C9⁶⁰. These studies also showed that FK506 binds to FKBP in a single *trans* conformation, although the unbound ligand exists in organic solution as a 2:1 mixture of *cis* and *trans* amide bond rotamers.

The support for twisted amide bond comes also from the solid state studies on FK506 and rapamycin where the dihedral angle between C8-

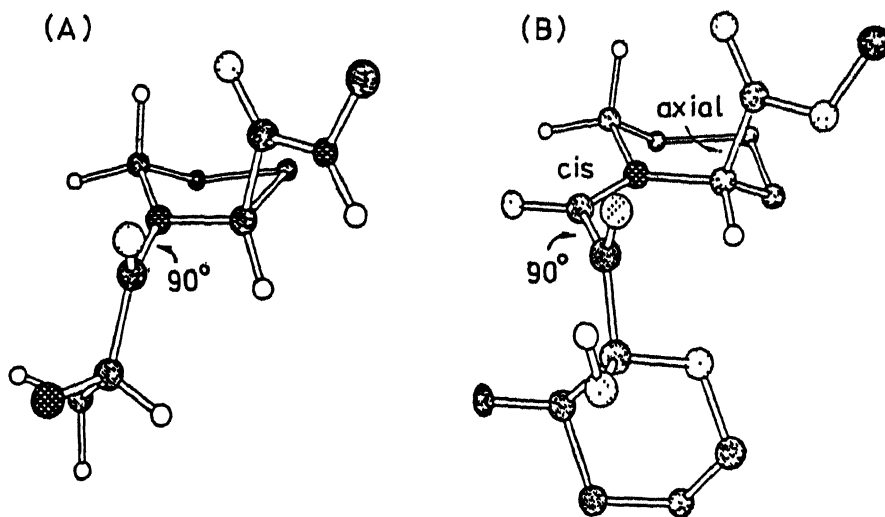


Fig 8 (A) Model of twisted peptide-prolyl amide bond portion of FK506/rapamycin showing a 90° dihedral angle between N7-C8-C9-C10. (B) Substructure of FK506. (adapted with permission from S L Schreiber *et al.* *J Org Chem* 55 (1990) 4984 Copyright © 1990 American Chemical Society).

C9 bond is approximately 90° . This conformation is also maintained in the complexes of FKBP with both the ligands. A planar N7-C8 amide group along with the 90° dihedral angle between C8 and C9 places the keto carbonyl roughly perpendicular to the plane of the pipercolinyl ring. The pipercolinyl ring most probably mimics the proline in natural peptide substrates. Therefore, the keto carbonyl of FK506 or rapamycin is in the same position as would be a twisted amide carbonyl group of a peptide, undergoing rotamase catalysis. Thus the perpendicular keto carbonyl group of FK506 and rapamycin allows the ligand to mimic a transition state structure involving a twisted amide bond. The view of FK506 and rapamycin as twisted amide peptidomimetics was further elaborated by studies of substrate, specifically of FKBP to peptide, series succinyl-Ala-Xaa-Pro-Phe-(p-nitro) anilide^{66a}. It was found that peptides having Xaa with branched hydrophobic amino acids, Leu, Ile, Val were more favoured than other amino acids. Analysis of FK506 and rapamycin data shows that they bind to FKBP because of their ability to mimic a Leu-Pro dipeptide with twisted amide bond. Similar observation have been made for CsA, where C α -C1 bond of MeVal 11 appears to mimic a twisted amide bond.

The change in the structure of CsA and FK506 in free state and when bound to immunophilins is intriguing. Wuthrich *et al* in their commentary⁶⁷, note these observations on CsA as "Receptor induced conformation change of the immunosuppressant, cyclosporin A". Such changes have been observed in other systems, yet one is led to think that the structure of the drug is getting perturbed due to the requirement that they should fit tightly into the receptor binding sites of the immunophilins. These results seem to confirm the expectation arising from the accumulated knowledge that flexible molecules change their shapes to form optimal interactions with binding partners.

A practical consequence of such a situation is the frustration in attempting to design drugs by analogy to the structure of flexible unbound active substrate. Thus Jorgenson has commented on these results as "Rusting of the lock and key model for protein ligand binding"⁶⁸. Results on the structural studies of the analogues of the drugs and the receptors have been striking. These studies brought some more surprises to the fore and produced more

comprehensible results. Availability of water soluble analogues of both CsA and FK506 and their NMR investigation in aqueous solvents were revealing. Interestingly both [D-MeSer 3-D-Ser(O-Gly) 8]Cs⁶⁹ and FK506 analogue, [32-Arg] ascomycin⁷⁰, have structures in water that are very similar to the structure of CsA and FK506 bound to their respective immunophilins. These results suggest that the immunophilin bound conformation of these drugs largely preexist in aqueous solutions and is not induced by the protein as previously postulated. These studies also brought out a well known, yet forgotten fact (in the context of studies with these immuno-suppressants), that solvents do play a major role in deciding the structure of molecules and one should keep this in perspective before comparing the structure of the free drug molecule, with that bound to its receptor.

The structural studies of FK506 bound to a mutant FKBP have also been undertaken⁷¹. This mutant protein causes the ligand backbone near the C16 to move by several angstroms. FK506 appears to undergo shifts in the binding pocket, with the largest change occurring at the effector region of the drug, which is involved in the binding with CN. Denesyuk *et al.*⁷² made a computer modelling study to see why despite such dissimilar structures CsA-CyP and FK506-FKBP perform the same biochemical function. It was found that nine amino acids of CyP and FKBP have similar arrangements at their active site pockets. Also, FK506 and CsA have structural similarities where they come in contact with the active site. The resemblance is specially striking around MeLeu 9, MeLeu 10 in CsA and pipercolinyl moiety in FK 506.

It has already been mentioned in the beginning of this paper that HIV-1⁷³, which is a retrovirus, carrying its genetic information in RNA, causes the suppression of the immune system. One is, therefore, tempted to look for similarities between the mode of action of immunosuppressant drugs and HIV. Recent discovery, that CyP binds to the Gag poly protein (the poly proteins are cleaved by protease, which produce functional proteins of the virus) of HIV-1⁷³ and is specifically incorporated in HIV-1 virions⁷⁴, being required for their infectious activity, has sparked great interest. NMR spectroscopy has shown⁷⁵ that the heptapeptide, Ser-Glu-Asn-Tyr-Pro-Ile-Val, a model of an HIV-1 protease cleavage site in the Gag poly protein is a substrate of CyP. The *cis-trans* conversion rate of

the heptapeptide is enhanced by CyP. It raises the interesting possibility that CyP may be functioning in the HIV virion to catalyze the generation of the conformers in the poly proteins necessary for cleavage by HIV protease. These observations have raised many questions on several aspects of immunology, protein folding and retrovirology, specifically the question whether Gag protein of HIV-1 is also an immunosuppressant. CsA was found to compete with Gag for binding to CyP and led to a speculation whether the Gag protein has effects similar to that of CsA. It was shown that CyP-Gag complex does not bind to calcineurin, suggesting HIV-1 protein may mediate immunosuppression by other mechanism. One possibility is Gag protein disrupts the binding of CyP to a natural ligand²¹ and thereby influences T cell activation. Another important finding was that the virus that contained less CyP, due either to treatment with drugs or mutation, was less infectious and thus gives scope for therapeutic intervention. This leads to a suggestion that for a cyclophilin depleted virus the replication gets blocked between the reverse transcriptions of the RNA and integration of the DNA into the host cell genome⁷⁶. It seems reasonable to investigate whether cyclosporins, in particular non-immunosuppressant analogues, can be used for treatment of AIDS. With this notable observation that immunosuppressants, the drugs as well as the HIV, compete with each other, it would be interesting to discuss therapies of AIDS and the applications of NMR spectroscopy in the advancement in this area.

AIDS and Structure of Reverse Transcriptase Inhibitors

HIV infects the T4 lymphocytes, which play a central role in regulating the immune system⁷⁷. The resulting depletion of T4 cells, the hallmark of AIDS, leave the patients vulnerable to opportunistic infection that would not harm a healthy person. Even today, almost after two decades of the first observation of AIDS, efforts are going on to find an effective vaccine for the prevention or an efficacious drug for the cure of the disease. The triple combination therapy proposed in 1995 appears to be extremely successful and has brought tremendous cheer in the AIDS community. Several studies have shown that the potent combination of the drugs can reduce the amount of

HIV in blood to levels almost below the detectable limit of most sensitive assays. It has recently been discovered that this therapy also dramatically reduces the amount of HIV RNA in lymph nodes, the hiding place of the virus. These results are dramatic. Though, HIV is even more impervious to attack when it transforms its genetic materials from RNA to DNA, weaves itself into the host genes, and does not make new viruses. This strategy allows it to escape from the drugs. Duration of the disease would depend on how long cells harbouring HIV DNA survive, how many infected cells exist in an infected person and how much new viruses they can produce. Based on the computer model, Ho *et al.*⁷⁸ suggest that several years of treatment with a 100% inhibiting anti-HIV regimen might be able to eliminate all remaining viruses. As of now however, it would be wrong to believe that a cure of AIDS is close by. The understanding of how HIV causes diseases can be converted into treatment strategies. Several strategies in use to inhibit the HIV are discussed below and various sites of attack by the drugs are⁷⁷:

- 1 Absorption of the virus to the cell membrane, which depends on the interaction of the viral envelope glycoprotein gp120 and two cellular receptors CD4 and CCR5 (cysteine-cysteine chemokine receptor 5) which are present on the surface of a subset of T-lymphocytes and certain other immune cells.
- 2 Transcription of the viral RNA to proviral DNA by the RT and degradation of the residual RNA by ribonuclease H.
- 3 Integration of the retroviral DNA into the host DNA. This is a two step process. The first one, termed as 3' processing, involves removal of two nucleotides from each 3' end of the reverse transcribed viral DNA. In the second step, called the DNA strand transfer, the resulting 3'-OH end of the viral DNA are covalently joined to newly created 5' ends in the target DNA.
- 4 Expression of the viral genes by the regulatory proteins such as the *trans* acting transcriptional activator (tat).
- 5 Proteolytic cleavage of the viral precursor proteins by the viral proteinase, which itself gets auto-catalytically cleaved from precursor protein.
- 6 Glycosylation of the viral glycoproteins, an

intermediate step between the first and second rounds of glycosylation requiring trimming of the terminal glucose and mannose moieties by glycosidases and mannosidases respectively.

The target that has received maximum attention is the synthesis of viral DNA by the enzyme RT. This strategy is attractive because it involves a step that is unique to retroviruses. The large size of RT (≈ 50 kDa) forbids the study of the enzyme structure by detailed NMR spectroscopy. However structural study on the inhibitors in solution have been of great relevance. The natural nucleosides and nucleotides, that are essential for the viral DNA chain to grow, have hydroxyl groups at 3' position. 3'-hydroxyl group acts as the site of attachment of the next nucleotide of the growing DNA chain. Any break in the chain or in chain growth results in the inhibition of viral replication. Action at this stage generated the first AIDS drug 3'-azido-2',3'-dideoxythymidine (AZT) and several subsequent FDA approved drugs come from the 2',3'-dideoxynucleoside (ddN) class of compounds⁷⁷. In these molecules 3'-hydroxy group is absent and is replaced by a group that does not permit formation of phosphodiester linkages. AZT, approved as the first AIDS drug about a decade back, was found to reduce the morbidity and mortality involved with HIV infection. It is a prodrug and like other nucleosides, gets phosphorylated to AZT triphosphate (AZTTP) by a series of kinases (that usually phosphorylate thymidine). AZT-monophosphate (AZTMP) is first formed by thymidine kinase and two phosphates are then added by the sequential action of thymidylate kinase and nucleotide diphosphate kinase to form AZTTP, the active moiety of AZT.

AZT inhibits the action of RT by two mechanisms (i) AZTTP preferentially attaches to the nucleotide binding site and competitively inhibits the binding of deoxythymidine-5'-triphosphate, thereby preventing its attachment to the growing chain of viral DNA; (ii) AZT acts as a chain terminator after AZTTP is added to the growing viral DNA chain. Because of the presence of azido group at the 3' position 5',3'-phosphodiester linkage cannot be formed and viral DNA synthesis stops.

All ddN analogues possibly act in a similar fashion as AZT, implying that they must be phosphorylated intracellularly to their 5'-

triphosphate analogues before interacting with the RT. ddNTPs have about 50 fold greater affinities for the HIV RT than the corresponding natural 2'-deoxynucleoside- 5'-triphosphate (dNTP). Thus ddNTPs may act as either competitive inhibitors, i.e. prevent the incorporation of the natural substrate (dNTP) or alternate substrates and thus get incorporated in the growing viral DNA chain, leading to the DNA chain termination.

The difference in the anti HIV activity of different ddNs could be attributed to (i) difference in the rate (or extent) of phosphorylation to their 5'-triphosphates; (ii) difference in the affinity of these 5'-triphosphates for the HIV RT; (iii) difference in their ability to influence pool sizes of the substrates (dNTPs), they compete with. Increasing evidence suggests that the relative ability by which 2',3'-dideoxynucleosides generate their triphosphates intracellularly, correlates well with their activity. Recent experiments have thrown more light on the mechanism of AZT inhibition of the RT⁷⁹. Although the AZT conversion to AZTTP is facile, conversion of AZTMP to AZTTP is inefficient resulting in the accumulation of AZTMP. As AZTMP binds to the thymidylate kinase with high affinity, an increase in concentration of AZTMP antagonizes the normal activity of thymidylate kinase and thymidine monophosphate (TMP) conversion to thymidine triphosphate (TTP) becomes inefficient.

Cellular toxicity and viral resistance limits the efficacy of AZT. Subsequently other nucleoside based RT inhibitors like 2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytidine (ddC), 2',3'-didehydro-2',3'-dideoxythymidine (d4T) and 3'-thiacytidine (3TC) were approved as drugs making them the first of the five AIDS drugs. These drugs and some other protease inhibitor drugs are often to be used in combination with AZT in the therapy for best results⁷⁹.

Various methods like X-ray diffraction, NMR spectroscopy, Laser-Raman spectroscopy and theoretical calculations have been used to study the molecular structure of ddNs. NMR study of several ddNs have been performed in solution and certain trends have emerged⁸¹⁻⁹⁰. Nucleosides show high conformational flexibility primarily involving rotations about single bonds and the sugar pucker⁹¹. The important rotations are about the exocyclic C4'-C5' bond and the glycosyl bond. The dihedral angles γ and χ characterize the orientation of

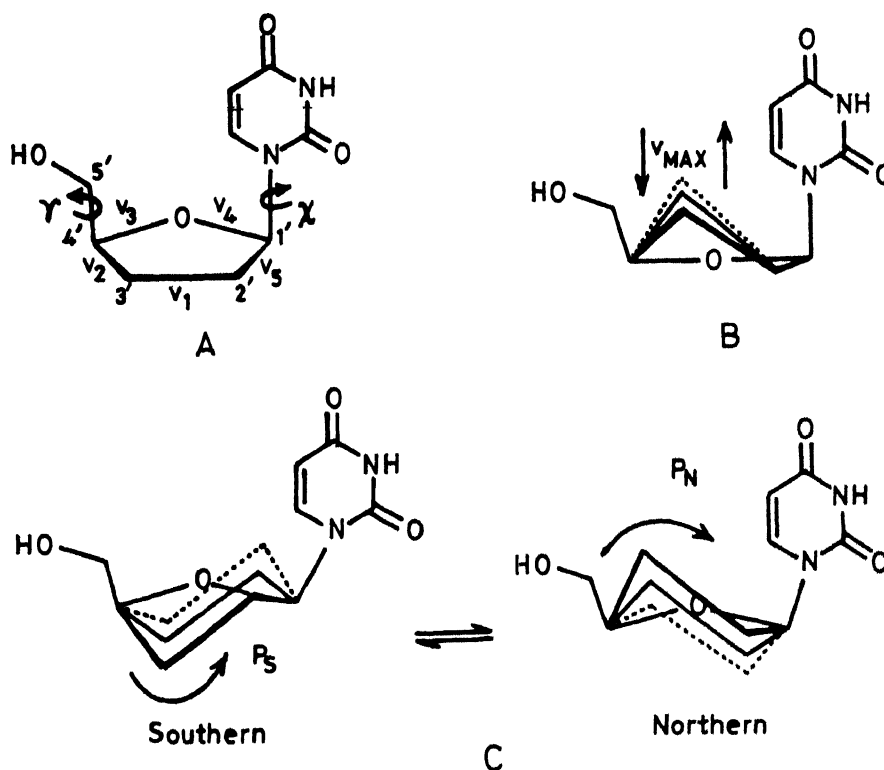


Fig. 9 Conformation of a dideoxynucleosides. (A) Definition of dihedral angles γ and χ . The angles ν_1 - ν_5 are the five endocyclic torsion angles which are related to the exocyclic torsion angles derived from NMR coupling constants (B) Definition of the maximum puckering angle ν_{max} . This is the maximum deviation from planarity for the ring and is equal to the largest value of the five endocyclic torsions. (C) Definition of the pseudorotational angle P . P varies from 0 - 360° , and as it increases the ring pucker progresses through alternating twist and envelope forms.

hydroxymethyl group and the base respectively. The sugar ring geometry is described by using the concept of pseudorotation⁹², in which the five endocyclic torsion angles are related to puckering amplitude (ν_{max}) and phase angle of pseudorotation (P) (Fig. 9). NMR studies of sugar rings of nucleosides and nucleotides show the presence of a fast equilibrium between two broad classes of conformers. These two classes are referred to as the *N*-type, with phase angle $P(N) = 0^\circ \pm 90^\circ$, puckering amplitude $\nu_{\text{max}}(N)$ and population X_N and the *S*-type with phase angle $P(S) = 180^\circ \pm 90^\circ$, puckering amplitude $\nu_{\text{max}}(S)$ and population X_S ($X_S = 1 - X_N$). The observed couplings are averaged over two sugar pucker, characterized by $P(N)$, $P(S)$, $\nu_{\text{max}}(N)$, $\nu_{\text{max}}(S)$, X_N and X_S .

It is found that conformation around C4'-O5' have a preference for γ^+ conformers while the least populated conformer is γ^- and the glycosidic angle prefers an *anti*-conformation. These results are not

very different from those for natural nucleosides. X-ray diffraction study also provide qualitatively the same results. The sugar ring geometries show interesting variations depending on the substituents in the ring. In case of 3' substituted ddNs the population of the two conformers depend on the electronegativity of the substituent. While AZT shows almost equal populations of *N*-type and *S*-type conformers, ddI, ddC and other similar nucleosides (without a substituent at 3') show predominance of *N*-type conformers. Natural nucleosides and 3' fluoro-substituted ddNs, on the other hand show predominance of the *S*-type sugar conformation. These results follow a pattern consistent with *gauche* effect⁹³, which predicts the preference of *S*-type conformers for 2'-deoxyribose sugars. When the substituents at 3' are more electronegative, the *gauche* effect is reinforced and preference for *S*-type sugar pucker is enhanced, while a less electronegative group leads to predominance of *N*-type sugar pucker, as is

observed in unsubstituted ddNs. X-ray results of Van Roey *et al.* on several of the ddNs have shown the presence of unusual sugar pucker of C3' exo/C4' endo⁹⁴. They have implicated the anti HIV activity of some of the ddNs to this sugar ring conformation. NMR studies, however, do not support such a conclusion. The value of $\nu_{\max}$ from NMR and X-ray diffraction studies for these nucleosides is around 35°.

From these results no definitive conclusions about the geometrical requirements of a RT inhibiting AIDS drug could be drawn. Since ddNs are prodrugs, they are converted to the dideoxynucleotides, which in turn interact with the RT. In view of these, the NMR studies of dideoxynucleotides have also been undertaken⁹⁵. AZTTP did show some significant variation in the population of the sugar ring conformation compared to AZT. While for AZT the value of X_S was 0.52, which increased to 0.75 for AZTTP, a value very similar to that for natural nucleotides. Such a variation in sugar conformation was, for example, not observed for other ddNTP. The ddNTPs investigated showed predominance of *anti* conformers about the glycosidic bond, γ^+ about the C4'-O5' bond and β^+ about C5'-O5' bond. These conformations are similar to those obtained for other natural nucleotides and reflect more rigid structure for the dideoxynucleotides compared to the dideoxynucleosides.

Due to large size of the receptors, difficulty in getting labelled receptors or low binding affinity of the drug to the receptor, it is not always feasible to determine the complete structure of the protein-ligand complex. In such cases it is often possible to derive information about the bound ligand conformation using transferred NOE (TRNOE) technique⁹⁶. This is suitable when exchange between free and bound ligand is fast on the relaxation time scale. Exchange of the NOE from bound ligand to free ligand occurs, allowing the information on bound ligand to be obtained from the readily detected free ligand signal. The application of TRNOE has been made on the RT bound conformation of AZTTP⁹⁷. These studies show that the bound conformation of sugar ring is not the normal C2'-endo or C3'-endo conformer, instead it is C4'-exo. However the RT bound conformation of thymidine triphosphate was also identical to the RT bound AZTTP conformation. This leads to a suggestion that the similarity in

molecular geometries enable AZTTP to competitively inhibit the binding of TTP to RT and may be responsible for the success of AZT as an AIDS drug.

Structure of HIV Protease-Inhibitor Complex

The design and development of HIV protease inhibitors, the other class of AIDS drugs, have revolutionized AIDS therapy and brought a ray of hope in the gloomy world of AIDS patients. In the late 1995 and early 1996 some of the protease inhibitors were approved as drugs (bringing the total number of drugs approved till date for AIDS to 11) and a combination therapy using two RT inhibitors, AZT and 3TC, and a protease inhibitor, zidovudine (one of the four approved by FDA, others being saquinavir, indinavir and nelfinavir), has shown excellent results⁹⁹. This invariably brought the number of HIV in the blood almost below the detectable limit. The full implications of this therapy, which can fail for a variety of reasons, specially due to the intricacies of administering the drugs at a strictly prescribed time, over a long duration, are yet to be realized. Keeping HIV at bay is a daunting task. Even the best treatments have a difficult time completely suppressing viral replication, which gives drug resistant mutants a chance to appear. Thus fully rebuilding an HIV ravaged immune system is a tall order⁹⁸.

The development and design of protease inhibitor drugs have used the lessons learned from research in renin inhibitors and their crystal structures. Three dimensional structures obtained from X-ray diffraction⁹⁹ investigation showed gross structural similarities between HIV protease and pepsin family of mammalian proteases. It was obvious from detailed structures that there are significant structural differences between them, which could be exploited to design protease inhibitors, specific to the viral enzyme. Crystal structure of several HIV protease and HIV protease-inhibitor complexes have been obtained. Such studies were mainly responsible for the developments of the protease inhibitor drugs for AIDS^{100,101}.

HIV protease, from the family of aspartyl protease, a homodimer with 99 amino acids in each monomer and molecular mass of 22.2 kDa, is ideal for NMR investigations. Structure of HIV protease in solution by NMR is not available as yet, despite the fact that NMR technique is suitable for this

enzyme. The difficulty has been due to the rapid autolysis of the enzyme. The structure of a potent cyclic urea based inhibitor (DMP323)-protease complex, however has been obtained by NMR recently^{102,103}. The target of an inhibitor design is based on an important difference between the viral and mammalian proteases, that a structural water molecule is present, which coordinates the tips (residues 51, 52) of the viral protease flaps to the inhibitor. The DMP323 was rationally designed as the inhibitor of protease, ($K_i=0.27$ nM), as this cyclic urea analogue displaced the water molecule. Indeed the conserved water molecule was not seen in the HIV protease- DMP323 crystal structures¹⁰¹ as well as in the NMR studies¹⁰⁴.

The stereospecific assignments of several methylene and methyl protons were obtained by the use of various 3D and 4D techniques¹⁰³. The ¹H spectra of DMP323 bound to protease were obtained by a combined use of ¹³C edited 2D NMR spectra. Intramonomer nOes were distinguished from intermonomer nOes by comparing the results from NMR spectra of fully ¹³C, ¹⁵N labelled protease with spectra of a isotopic heterodimer which has 50% of the monomer molecules labelled with ¹³C, ¹⁵N. Special editing techniques were used for specially selecting intermonomer nOes^{105,106}.

The tertiary structure of the complex was determined by using a systematic three step approach¹⁰³. First step was to obtain an approximate monomer structure followed by the dimer structure and lastly the structure of full protease-inhibitor complex was derived. A preliminary monomer structure was obtained using intramonomer distance and dihedral angle constraints. For finding the dimer structure intermonomer nOe and hydrogen bonds were obtained. Fig. 10 shows the topology of the four stranded antiparallel β sheet, formed by the N-terminal β strand (the first β strand, $\beta 1$) and C-terminal β strand (the tenth β strand, $\beta 10$) of the monomer units along with the nOe and hydrogen bonds. This $\beta 1$ - $\beta 10'$ - $\beta 10$ - $\beta 1'$ (prime used for distinguishing one monomer from the other, as well the amino acids in one residue are numbered 1 to 99 while in the other from 101 to 199) arrangement is same as observed in the X-ray crystal structure¹⁰⁰. In addition to the terminal regions, intermonomer nOes are observed between residues at 25-27 and 49-54, showing the proximities of these residues of the two monomers. Using several

of the intramonomer and intermonomer distance constraints, the dimer structure without inhibitor was obtained

The last step in obtaining the structure of protease-inhibitor complex was to include the data on the inhibitor. Conformational studies¹⁰¹ predicted that the 7-membered cyclic urea ring conformation of DMP323 (Fig. 11A) prefers the pseudo-diequatorial hydroxyl groups and pseudo-diaxial benzyl groups (P1-P1'). This was confirmed by X-ray diffraction studies of DMP323 and protease-DMP323 complex. The NMR data on protease-DMP323 complex is in agreement with the X-ray single crystal structure of DMP323 and protease-DMP323 complex¹⁰¹.

The dimer structure derived by NMR, without the inhibitor, was used to obtain the final structure by positioning the urea oxygen along the symmetry axis of the molecule and the inhibitor diol moieties close to Asp 25 and Asp 125 carbonyls in conformity with previous X-ray¹⁰¹ and NMR^{102,104,107,108} studies. This crucial step enabled distinguishing several drug-dimer nOes and resolved many ambiguous assignments. The structure of protease-inhibitor complex obtained using distance and dihedral angle constraints is shown as 23 superimposed structures in Fig. 11B. Fig. 11C shows the structure in the form of ribbon diagram.

This study provided the first solution structure of HIV protease (with or without the inhibitor) which closely resembled the X-ray single crystal structures of several protease and protease-inhibitor complexes. The first four residues form the outer part of the interfacial four stranded β -sheet ($\beta 1$, $\beta 1'$), involving terminal residues, that stabilizes the dimeric structure. There are ten β strands involving residues 1-4, 9-15, 19-25, 30-35, 45-49, 53-60, 62-65, 70-78, 83-86 and 96-99 and a single helix of the monomer at residues 87-95. Several turns and hair-pins join the regular secondary structures. The agreement between NMR and X-ray structure^{100,101} is generally good, though distinct variations exist locally in several regions of the sequence that appeared to be important for functions. While the polypeptide chain is found to be disordered at residues 66-69, which show both type I' and type II' β turns, the residues 38-42 show large amplitude internal motions on 10^{-9} - 10^{-12} s time scale.

In contrast to rapid motions for residues 38-42,

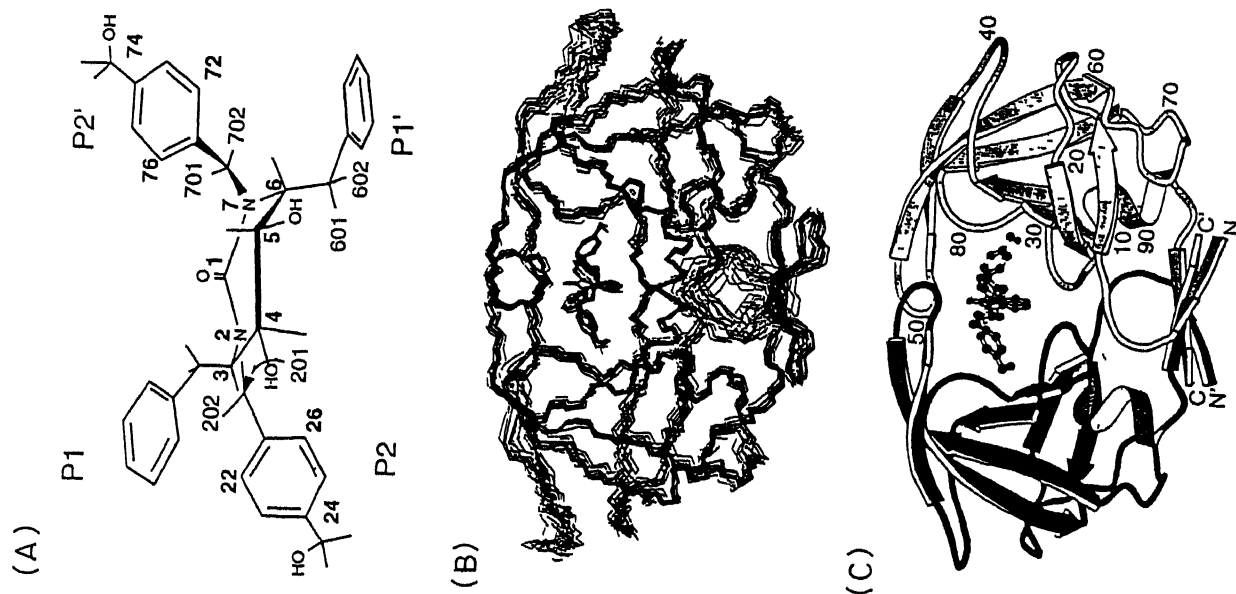


Fig 11 (A) Chemical structure and conformation of DMP 323 (B) View of the superimposed heavy atom coordinates of 23 NMR structures of protease-DMP323 complex (C) Ribbon diagram of the restrained minimized average NMR structure of protease-DMP323 complex (adapted with permission from T Yamazaki *et al Protein Science* 5 (1996) 495 Copyright © 1996 The Protein Society)

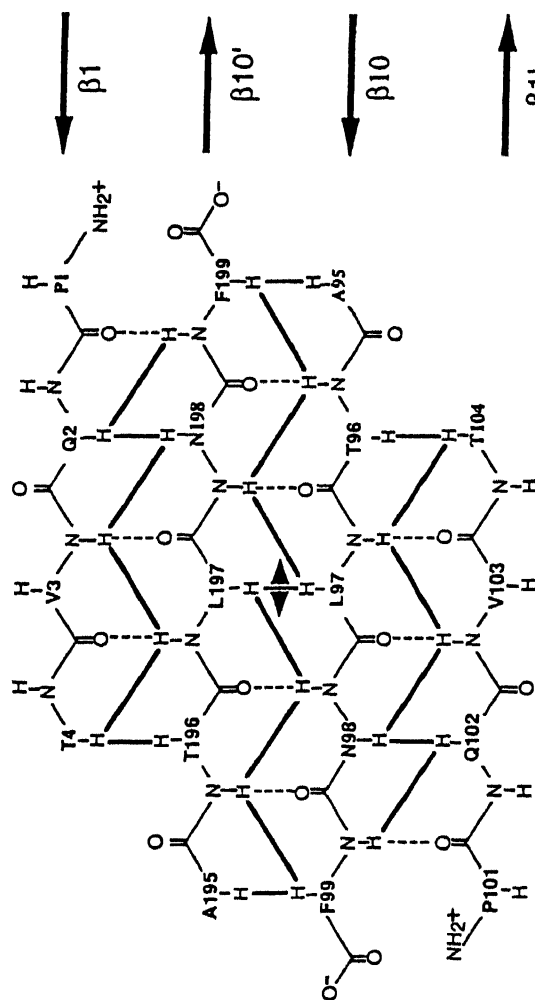


Fig 10 Intermonomer β sheet topology of the HIV protease. Residues in the first monomer unit are numbered from 1-99 while in second monomer from 101-199 (the strands in this monomer are labelled by the prime superscripts) (adapted with permission from T Yamazaki *et al Protein Science* 5 (1996) 495 Copyright © 1996 The Protein Society)

Thr 4 and Leu 5 execute motions in 10^{-3} - 10^{-6} s time scale¹⁰⁸. The primary site of autolysis of the protease is Leu 5-Trp 6 peptide bond, and flexibility of this region may be important for viral function, specially because of the suggestion that the rate of cleavage of this bond may regulate the activity of the protease¹⁰⁹. The tips of the flaps of protease dimer, linking Ile 50 and Gly 51 with β turn, also execute slow motions on 10^{-3} - 10^{-6} s time scale. This region is also involved in an important functional role in stabilizing the enzyme-substrate complex. The NMR data is not consistent with a single type of turn at residues 50-53. Low temperature studies also failed to freeze into a single conformation. It is presumed that, on crystal formation a single conformation is stabilized in the solid state. The flexibility of the residues 38-42 and 50-51 may be important in facilitating substrate binding and product release, because these residues make up the respective hinges and tips of the protease flaps.

The flexibility of the protease, so significantly evident in the protease-DMP323 complex, suggests that the HIV protease alone (without the drug) is very flexible. Such a suggestion has already been made by the X-ray studies of uninhibited protein, which shows open flaps that permit access to the active site involving Asp 25. Recently Kent *et al.* carried out NMR studies¹¹⁰ on chemically synthesized HIV protease labelled with ^{13}C at the catalytically essential Asp 25 in the presence of an inhibitor pepstatin, a mimic of the tetrahedral intermediate formed in the enzyme catalysis. It was interesting to note that in the protease dimer, one of the Asp 25 was protonated while the other was deprotonated. Similar observation was made by Torchia *et al.* using an asymmetric protease inhibitor¹¹¹. The direct observation of the chemical properties of the catalytic apparatus of the enzyme provides concrete information on which to base the design of improved HIV-1 protease inhibitors. Although autolysis precludes studies of the wild type protease by NMR spectroscopy, mutants with full activity and much higher stability of the enzyme are now available¹¹². This will enable the characterization of the structure and dynamics of the free enzyme by NMR methods.

Structure of Peptides from V3 Loop of gp120

The best way to combat a disease is to prevent it. Vaccination is the simplest, safest and most

effective form of prevention of the disease. Vaccines have achieved great success against viruses. Stupendous effort is on to develop an effective vaccine against the HIV and it remains, perhaps, the most formidable challenge for the virologist today⁹⁸. Such efforts have not succeeded so far mainly because of the devious nature of the virus, which can hide in cells, change the composition of its coat protein and install its own genes within the genes of its host. Lack of a good animal model also does not permit investigations to combat these ploys of the HIV.

Most of the vaccine development effort has focused on gp120, the envelope glycoprotein with a mol weight of about 120 kDa. Due to heavy glycosylation, most of gp120 is obscured from immunological sight by the cloud of sugars. The immunogenic properties of gp120 bring out two of its features; one is the binding site of the host T4 cell receptor molecule CD4 and other a loop, which is highly immunogenic¹¹³. The main binding site of the HIV-1 neutralizing antibodies, the principal neutralizing determinant (PND), is located within the disulfide linked third hypervariable loop in the V3 region of the gp120¹¹⁴⁻¹¹⁷. Fusion between the cell surface and the virus also results due to a proteolytic cleavage of a peptide bond within the V3 loop. Sequence of PND from several HIV-1 isolates revealed that certain amino acid residues are relatively conserved¹¹⁸. The sequence Gly-Pro-Gly-Arg-Ala-Phe (GPGRAF) occurs in many PNDs and the antisera directed against it were shown to neutralize multiple isolates¹¹⁹. The conserved sequence has, therefore, been the target of extensive studies for peptide based vaccine development¹²⁰⁻¹²². Several gp120 peptide based vaccines are already at various stages of clinical trials. Despite the fact that the results are disappointing so far⁷⁹, possibly due to the hypervariability, existence of several conformationally distinct forms or low accessibility of the envelope protein, very serious efforts are being made to find a vaccine for AIDS. The recent discoveries of the chemokines and their receptors, CCR5 and CXCR4 for the HIV will definitely open up new avenues for the development of drugs and vaccines against AIDS^{123,124}.

Study and comparison of the conformation of these immunogenic peptides with those of the antibody bound peptides and of the corresponding parts of the proteins could be of great help in

understanding the molecular basis for the cross reactivity of anti-peptide antibodies with the native proteins. Such studies are also likely to throw light on the contribution of various structural features to their antigenicities. V3 loop is typically 35 amino acid long, with residue number 1 and 35 as invariant cysteine involving disulfide bridge. Conformational studies of several PND peptides in the V3 loop have been carried out by NMR¹²⁵⁻¹³³, specially to understand the nature of the conserved GPGRAPH sequence and the role of the disulfide linked loop. The following synthetic peptides containing upto 40 amino acid residues (referred to with single letter code of amino acids), mostly with the sequence from the common HIV-1 isolates, have been studied (numbers inside the brackets are references):

RP70	- INCTRPNYNK RKRIHIGPGR AFYTTKNIIG TIRQAHCNIS ¹²⁵
RP142	- YNKRKRIHIG PGRAPHFYTTKN IIGC ¹²⁵
RP342	- IHIGPGRAPHY TC ¹²⁵
RP135	- NNTRKSIRIQ RGPGRAPHFTI GKIG ¹²⁶
P547	- NNTRKSIRIQ RGPGRAPH ¹²⁶
P344	- GGRAPHFTIG KIGGKK ¹²⁶
T1RF	- KQINMWQEV GKAMYATRP NNTRKSIHMG PGKAFYTTG ¹²⁷
T1CANO	- KQINMWQEV GKAMYATRP NNTRKSITKG PGRVIYATG ¹²⁷
P1	- CTRPNNTRK SIHIGPGRAPH YTTGEIIGDI RQAHC ¹²⁸
P2	- P1(D-Ala17) ¹²⁸
P3	- P1(D-Pro16) ¹²⁸
P4	- P1(N6-Glucose NAc) ¹²⁸
P5	- CTRPNNYK RK SIHIGPGRAPH YTTGEIIGDI RQAHC ¹²⁹
P6	- C(P5) ¹²⁹
P7	- Cc(CTRPNNTR TSITIGPGQV FYRTGDIIGD IRKAYC) ¹³⁰
P8	- Cc(CTRPNDNTR KSIPMGPGKA FYATGDIICN IRQAHC) ¹³¹
P9	- Cc(CTRPNNTR KSITKGPGRV IYATGQIIGD IRQAHC) ¹³¹
P10	- GGRAPH ¹³²
P11	- GGRAPHFC ¹³²
P12	- GGRAPHFGPGRAPH ¹³²
P13	- GGRAPHResin ¹³³

For peptides RP70, RP142, Chandrasekhar *et al.*¹²⁵ found a significant population of

conformations containing a β -turn at the highly conserved sequence GPGR, while for the shorter peptide RP342 the population of β -turn conformation was not significant. The other segments of these peptides showed conformational averaging. Zvi *et al.*¹²⁶ studied peptides RP135, P547 and P344. RP135, a 24 amino acid peptide corresponds to PND of IIIB isolates of HIV-1. Other 16 residue peptides include GPGRA sequence with N-terminal part of RP135 (P547) and C-terminal sequence of RP135 with GPGRA (P344). In RP135 the C-terminal part was found to exist in several transient turn like conformations referred to as "nascent helix" with a transient turn in the GPGR sequence. The conformation of shorter peptides was similar to those of the corresponding portions of RP135. Spicer *et al.*¹²⁷ studied two synthetic peptides taken from C4 and V3 domains of HIV-1 gp120. The segment taken from C4 domain in both peptides exhibits helical character with a reverse turn in the conserved GPGX (X=R or K) sequence. Griesinger *et al.*¹²⁸ studied synthetic peptide antigens, P1, P2, P3 and P4, containing 35 amino acids, from V3 region of the glycopeptide of HIV-1. In P2 a D-Ala was introduced at residue 17, instead of Gly, the i+2 position, for stabilizing the type II β turn. They also observed high propensity of type II β turn in P1, P2 and P4 (a glycopeptide) while P3 had a type I' β turn at G-D-Pro-GR. Nascent helices are observed in the C-terminal end of these peptides. Fig. 12 shows the stereoplot of folded type II β turn involving residues 13-20 of P1.

Gupta *et al.* also studied¹²⁹⁻¹³¹ several peptide sequences from the V3 loop and observed that inspite of the sequence variation, the conserved structure is located inside the loop. Cyclization of P5 results in an N-terminal loop between residues 2 to 9, stabilization of type II β turn at GPGR and formation of two turns at residues 23-26 and 30-33. This incipient helix, found in aqueous solution, shows propensity of a helix in less polar solvents. The structural rigidity and a desirable structure makes the cyclic peptide a better ligand for monoclonal antibodies than the linear form. Peptide P7, P8 and P9 also show very similar structural features with a GPG(R/K/Q) crest in the centre of the neutralizing domain, two extended regions flanking the central crest and a helical segment in the C-terminal region. These results lead us to study¹³² much smaller peptides P10, P11

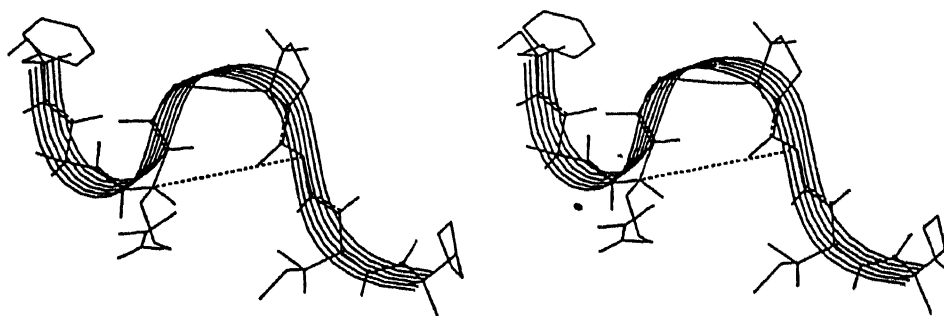


Fig 12 Stereoplot of folded type II β conformation of P1 (amino acids 13-20) (adapted with permission from C Griesinger *et al Eur J Biochem* 237 (1996) 188 Copyright © FEBS 1996)

and P12 and look for some incipient structure in them. Even these small peptides showed propensity of a turn in GPGR in DMSO solution. While P10 and P12 seem to have both type I and type II β turns, P11 showed propensity of only type II β turn. In presence of an appropriate receptor these peptides may take the required turn conformation and can be antigenic. The resin bound conformation of GPGRF was also found to be folded¹³³

Using NMR, Zvi *et al* have also attempted to map the binding of an antibody and a specific antibody binding epitope from an HIV-1 III B peptide¹³⁴. More explicit and detailed results were, however, obtained from the X-ray study of a complex of peptide RP142 with Fab fragment of a neutralizing antibody¹³⁵. It was found that the tip of V3 loop with GPGRFY, shows a multiple β -turn conformation. GPGR has a type II β turn followed by type III and type I β turn for GRAF and RAFY residues respectively. Some of the NMR results on peptides from V3 loop with β turn at GPGR and a "nascent helix" conformations are consistent with these X-ray studies.

Despite the conserved PND sequence, the hypervariability of V3 loop region possibly leads to altered conformation and thus the HIV antibody recognition of different V3 peptides is controlled by the primary as well as secondary structure of the antigen. It therefore appears that the vaccine development to kill HIV requires fresh line of thinking and some novel approaches will have to be devised. A renewed effort is being made in this direction, involving cytotoxic T lymphocytes (CTL)¹¹⁹, which play an important role in the body's natural defence, as they search out and destroy virus infected cells. Other approaches

involving genetically engineered viruses, specially the vesicular stomatitis virus (VSV) with CD4 and CCR5 receptors at its surface, have shown great promise¹³⁷.

Structure of HIV Integrase

¹H NMR spectroscopy has also been used to study the structure of the fragments of integrase and other structural proteins of the HIV. HIV integrase is responsible for the insertion of a DNA copy of the viral genes into host DNA, an essential step in the replicative cycle of HIV. HIV integrase comprises three functional domains. The central core domain, consisting the catalytic site, is flanked by a small N-terminal zinc binding domain and C-terminal DNA binding domain. While the catalytic core domain can carry out a simple polynucleotidyl transfer, termed disintegration, all three domains are required for the 3' processing and DNA transfer activities, that accomplish integration of the viral genome. Thus even the N- and C-terminal domains are potential useful targets for rational drug design for AIDS. NMR studies have been carried out on the N-terminal and C-terminal domains^{138,139}. The C-terminal domain (residues 220-270) of HIV-1 integrase is a dimer in solution. Each subunit consists of a five-stranded β -barrel with a topology very similar to SH3 domains, which are found in numerous proteins¹⁴⁰ involved in signal transduction. The N-terminal zinc binding domain (residues 1-55) of integrase contains a His₂Cys₂ motif that is conserved in all retroviral integrases. It binds one equivalent of zinc and is necessary for full integrase activity. The isolated domain is unstructured in the absence of zinc, but folds into a dimer with high α -helical content in the presence of zinc. Each monomer has four helices with zinc

tetrahedrally coordinated to His 12, His 16, Cys 40 and Cys 43. This fragment exists in two interconverting conformational states, differing in the coordination of two histidine side chains to zinc. The monomer fold of the N-terminal domain is remarkably similar to that of a member of helical DNA binding protein, containing a helix-turn-helix motif. We would not discuss these results in further detail as they have not yet been exploited in AIDS therapy. Similarly we have not included in this chapter the NMR studies on other structural proteins of HIV.

Outlook

There is considerable evidence that paradigm shift is occurring now in the area of drug discovery¹⁴¹. Presently medicinal chemists are not only generating new structures, they are also designing more compounds with higher biological activity, higher selectivity, higher bioavailability and at the same time with lesser toxicity. NMR spectroscopy in combination with X-ray crystallography and appropriate theoretical methods for structural elucidation, constitute the best method for the development of structure activity relationship in drug design. Combinatorial chemistry¹⁴²⁻¹⁴⁵ and high throughput screening have been revolutionizing the drug discovery programmes for the past few years. Combinatorial chemistry is already supplementing the historical approach of medicinal chemistry, starting from natural products leads. Modern and fast screening methods yield oligomers and analogues with high receptor-binding affinities. Interestingly such screening can often be accomplished without precise information about the receptor. The combinatorial libraries result in large number of molecules, invariably obtained through solid-phase synthesis. Molecules bound to resins can exhibit biological activity and immunogenic properties. A major challenge has been to find out what these libraries contain. ¹H and ¹³C NMR methods¹⁴⁶⁻¹⁵⁰, using magic angle sample spinning (MAS) of organic molecules covalently attached to beads, are being developed. These allow non-destructive monitoring of solid-phase reactions and should play an important role in future developments in the pharmaceutical applications. Pursch *et al.*¹⁵¹ have used macrobeads, with particle size 400µm, based on resins with long flexible tentacles and tethers. Synthesis was carried out on a single macrobead.

Each step in the synthesis was followed by ¹H MAS NMR using a single bead, which makes the characterization of the building blocks of molecules in the polymeric support quite convenient and straightforward.

Recently Fesik *et al.* suggested an NMR based structure activity method (SAR by NMR)^{152,153}. This method involves screening of libraries of small molecules to identify, optimize and link components that bind to proximal binding sites of ¹⁵N labelled protein. The compounds were screened by monitoring perturbation of the amide chemical shifts of the labelled protein, upon the addition of protein ligands. Using this approach and combinatorial synthesis, Kessler¹⁵⁴ has identified two weakly FKBP binding ligands with association constants $K_d=0.1$ mM and $K_d=2$ mM. Linking these two ligands with various $-(CH_2)_n-$ groups resulted in the recognition of a molecule with great affinity ($K_d=19$ nM) with FKBP. It seems likely that, as combinatorial chemistry is applied more widely in drug discovery, the rules for molecular recognition will become more transparent making structure-based drug design as the method of choice. This will enable finding leads, and developing them into drugs two to three times faster than has been possible. Such studies may also have important applications in the development of vaccines.

We have presented a view of NMR application to some interesting and contemporary problems in the area of immunology. An attempt has been made to show how important is the knowledge of three dimensional structure of drugs, receptors and drug-receptor complexes, in designing an effective and efficacious drug.

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SPATIALLY ENCODED MR: IMAGING, VOLUME LOCALIZED SPECTROSCOPY, MEASUREMENT OF TRANSPORT

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The basic physics of magnetic resonance is briefly introduced, including the principles of spatial encoding, which is the main concern of this contribution. Simple examples of spin echo imaging are given for illustrative purposes. The interest in dealing with properties of multi-component systems – and especially of minor components therein – is emphasized. In this context the multiple quantum imaging experiment and the proton detected carbon-13 imaging experiment are discussed. An example of deuterium imaging in lyotropic phase is included. Volume localized spectral properties in such systems may be investigated by implementing multi-voxel multiple quantum spectroscopy as well as single voxel two-dimensional spectroscopy. Finally, a fast multi-echo diffusion measurement protocol is described. Quite clearly, the developments in spatially resolved MR permit the detailed characterization of properties at the molecular level as well as the measurement of ‘macroscopic’ distribution functions of such properties in heterogeneous systems.

Key Words : Magnetic Resonance; Spatial Encoding; Multi-Component Systems; Multiple Quantum Imaging; Indirect Carbon-13 Imaging; Single Voxel and Multi-Voxel Spectroscopy; Fast Diffusion Measurements

Introduction

Into its sixth decade after discovery, Magnetic Resonance (MR) has become a prime spectroscopic technique, with scarcely a branch of science left untouched by it—and yet with no signs of abatement in further developments of the field. Over the years, this field has grown from being the plaything of physicists, to becoming the staple of chemists, then morphing to the champion spy of molecular biologists, now the oracle of biomedicine and the powerful ally of chemical engineers.

Such striking success and infinite variety - which custom has failed singularly to stale! - arises, in a nutshell, from the circumstance that MR deals with intrinsic properties of elementary particles, but with an external handle that permits a breathtaking degree of manipulation, all accomplished with quanta of extremely small energy—typically smaller even than the energy of thermal fluctuations at room temperature. Indeed MR is the non-invasive, non-destructive technique par excellence.

To recap, magnetic resonance arises from the ability of resonant electromagnetic (em) radiation

to reorient the precessing magnetic moments of nuclei or unpaired electrons^a in molecular matter - and therefore their ‘bulk magnetization’ - when they are located in a steady (dc) magnetic field. One is dealing all the way with magnetic interactions here, be it with the magnetic field or with the magnetic component of em waves. At the same time, there are statistical distributions and thermal equilibria to take into account, owing to the extremely small energy differences between different orientations of the spin in a magnetic field. This is the main reason that one prefers to resort to a resonance experiment rather than a direct measurement of dc (static) susceptibility: the resonant measurement of high frequency susceptibility effectively amplifies the dc susceptibility by several orders of magnitude.

The combined effect of magnetic fields **B** and thermal equilibration processes may be expressed classically in terms of a simple master equation describing the time evolution of the ‘bulk magnetization’ **M** of the sample:

$$\frac{d\vec{M}}{dt} = \gamma(\vec{M} \times \vec{B}) - \hat{k} \frac{(M_z - M_0)}{T_1} - \hat{i} \frac{M_x}{T_2} - \hat{j} \frac{M_y}{T_2} \quad \dots (1)$$

^a Simply called spins, in the jargon of MR

The first term in eq. (1) describes the precession of the magnetization in the magnetic field, with its characteristic Larmor precession frequency given by:

$$\omega = -\gamma B \quad \dots (2)$$

In eq (2), γ is a constant termed the magnetogyric (or, somewhat less appropriately, gyromagnetic) ratio of the spin. The precession frequency in attainable dc fields falls typically in the radiofrequency (rf) or microwave region of the em spectrum.

The other three terms on the rhs of eq. (1) describe the return to thermal equilibrium of the spin system, following a disturbance. This process is generally governed by two distinct time constants, known as relaxation times, corresponding to the restoration of bulk magnetization M_0 in the direction of the magnetic field, and to the loss of precessional phase coherence perpendicular to this direction. These processes, incidentally, are packed with information concerning the interaction of the spin degrees of freedom among themselves and with other non-spin degrees of freedom of the object.

Note that the bulk magnetization is generally given in terms of a Curie susceptibility which depends on the number N of spins per unit volume, and their spin quantum number I :

$$M_0 = N\gamma^2\hbar^2 I(I+1) \frac{1}{3kT} B_0 \quad \dots (3)$$

The spin quantum number I can take the values 0, 1/2, 1, 3/2, ... , corresponding to $(2I+1)$ possible orientations of the spin in the magnetic field (given by magnetic quantum number $m = I, I-1, \dots, -I-1, -I$). Note further that eq. (2) describes not only the precession of spins in a dc magnetic field, but, in a suitable co-ordinate frame (termed the Zeeman interaction frame or simply the rotating frame), also describes the nutation of spins in a resonant em field. *Because the frequency of oscillation of resonant em radiation matches the Larmor precession frequency of the spins, the radiation appears static to the spins and is therefore able to reorient them with its magnetic field component.*

The role of resonant em radiation in re-orienting the spins - corresponding to unit change of their magnetic quantum number - results in a non-zero component of the magnetization of the sample transverse to the dc field: a transient non-

equilibrium situation, once the radiation is turned off^b. In this non-equilibrium situation, a signal voltage $S(t)$ is induced in the spectrometer detector coil:

$$S(t) \sim \frac{dM}{dt} \sim M_0 \omega \cos \omega t \sim N\gamma^3 B_0^2 \quad \dots (4)$$

It is interesting to note, especially, that signal intensities are directly proportional to N , the number of spins per unit volume. Noise voltages can normally be modelled as being white and proportional to the square root of the operating frequency, ie, $\sqrt{(\gamma B_0)}$. Since signal is a coherent phenomenon while noise is random, the signal-to-noise ratio may be improved by co-adding the results of successive identical experiments^c. It may also be pointed out that among nuclear isotopes, protons (^1H) have the highest detection sensitivity, because they have the highest γ (except for the radioactive ^3H nucleus, whose γ is some 5% higher still) while the γ of electrons is 660 times that of protons - and of opposite sign.

Molecular applications of MR spectroscopy rest largely on the small, but finite—and accurately measurable—differences in the precessional frequency of a given spin when embedded in different molecular environments, in terms of chemical shifts and couplings. Such precise high resolution (HR) measurements carried out in the time domain—capturing the response^d of the spin system to an impulse excitation with resonant em radiation—constitute not only the bulwark of MR, but also provide the basic framework for the rich tapestry of variations of multinuclear, multi-dimensional MR. The time domain response of the sample is then Fourier transformed (FT) to produce the desired frequency spectrum.

High resolution measurements, however, basically restrict MR investigations to homogeneous samples, preferably in solution^e. Indeed, it is one of the major concerns of HR MR to artfully

^bWe would also draw attention to the fact that resonant em radiation can under suitable conditions change the spin orientation in such a way as to correspond to a change of more than one unit in the magnetic quantum number.

^cSignal increases linearly with the number of repetitions, while noise increases as the square root.

^dThe so-called Free Induction Decay (FID)

^eDissolution, incidentally, is strictly speaking clearly an invasive procedure

remove any residual inhomogeneities of the object - and those of the MR spectrometer itself! Unfortunately, however, this excludes investigation of most natural and man-made objects. *Spatially encoded MR addresses the issue of directly characterizing interesting heterogeneous objects: seeking to address at once issues both at the macro and molecular levels, and lay bare the spatial distribution of properties of interest - such as MR spectra, relaxation rates, transport, or simply the spin concentration.*

Spatial Encoding

The key to spatial encoding of MR lies in the external handles of dc - or rf - magnetic fields. Consider first an object which contains a single molecular species that has but a single shift and no couplings: resulting in a single resonance line in the HR MR spectrum of the appropriate spin species. Simple examples of such molecular species would be H_2O (^1H), CS_2 (^{13}C), fluoranthene hexafluorophosphate (e^-), etc. If the dc field were to have different intensities at different spatial locations of the object, the Larmor frequencies of the spin would be correspondingly distributed in space:

$$\vec{B} = \vec{B}_0 + \vec{G} \cdot \vec{r} \Rightarrow \omega = \omega_0 - \gamma \vec{G} \cdot \vec{r} \quad \dots (5)$$

$$\vec{G} = \hat{i} \left(\frac{\partial B_0}{\partial x} \right)_{y,z} + \hat{j} \left(\frac{\partial B_0}{\partial y} \right)_{z,x} + \hat{k} \left(\frac{\partial B_0}{\partial z} \right)_{x,y} \quad \dots (6)$$

Here, the spatial dependence of the field intensity is described in terms of the gradient $\vec{G}$. At the simplest level, then, the impulse response of the spin system registered in the presence of a gradient (the so-called read gradient or frequency encode gradient) would ideally display a distribution of resonance frequencies. Each resonance frequency in the distribution may be considered as corresponding to the spins in one of a notional stack of planes defined perpendicular to the gradient direction, the intensities of the resonances being proportional to the number of spins in each such plane of the object. This experiment thus constitutes a measurement of the spatial distribution function describing the number of spins in such a notional stack of planes in the object — and as such is equivalent to imaging the object¹⁻³.

Imaging Experiments

Although the acquisition of one-dimensional profiles as described above can often be informative in materials applications, eg. in keeping track of kinetic processes, one is normally interested in a two-dimensional, if not a three-dimensional image of an object. It is possible to step up the dimensions in an NMR image by exploiting the fact that the spins have a phase^f memory (over a time-scale that corresponds to the spin-spin relaxation time T_2) and the MR receiver is phase sensitive^g. Subject to the phase memory of the spin system, therefore, one may encode simultaneously the desired information in two orthogonal directions, resulting in a phase that accumulates as a function of these so-called phase encode gradients, before reading out the spectrum in the presence of a read gradient in the third orthogonal direction. Systematic repetition of the experiment with independent incrementation of the two phase encode gradient amplitudes produces then a three-dimensional dataset which is a function of the two phase gradient amplitudes, as well as the read gradient. Three dimensional Fourier transformation of this dataset produces the desired 3D image^h. On the other hand, a two-dimensional image of a selected plane ('slice') of the object may also be produced by fulfilling the resonance condition only in the desired plane in order to select it, then phase encoding it in one of the directions defining the plane, followed finally by a frequency encode in the second orthogonal direction that defines it. A two-dimensional dataset now results upon repeating the experiment with incrementation of the phase encode gradient amplitude, permitting retrieval of the 2D image by a two-dimensional Fourier transform. Note that either way, the entire procedure is strictly non-invasive. Possible experiment protocols ('pulse

^fRecall that phase is just the integral over time of frequency.

^gPhase sensitive detection consists in multiplying the MR signal with a reference from the excitation source, followed by low pass filtration. Quadrature detection is commonly performed in MR, implying the phase sensitive detection of the MR signal in two identical channels which are fed reference signals that are identical but for a 90° phase shift with respect to each other

^hNote that three orthogonal gradients may be produced by employing three sets of orthogonal gradient coils. Further, the direction of a gradient may be changed at will by a suitable linear combination of currents in the three sets of coils

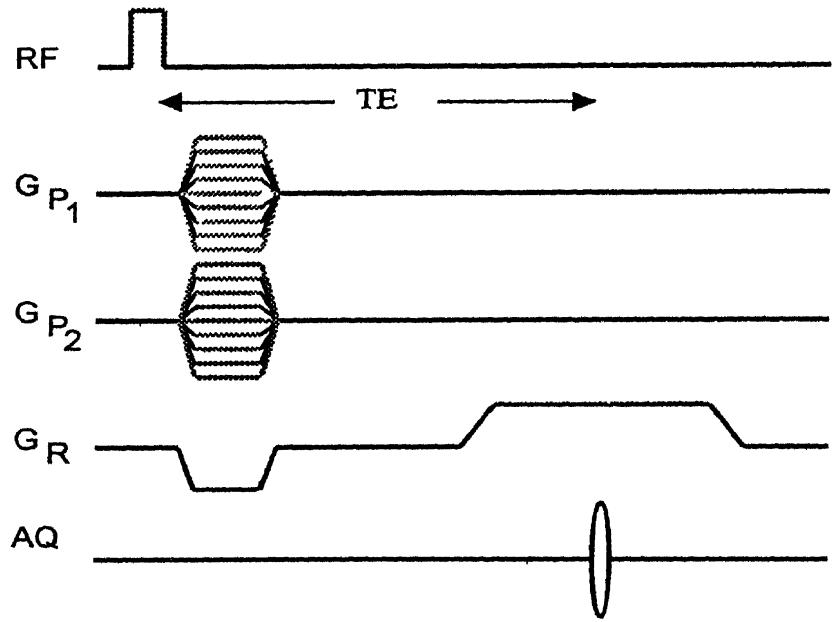


Fig 1 Pulse sequence for a three-dimensional imaging experiment. The x-axis of the figure represents time, while the y-axis represents amplitudes (of RF pulses, field gradient pulses and detected MR signals). The signal is acquired (*cf.* the AQ window) as a gradient echo (a pair of back-to-back FID's). The echo is produced in the above protocol by reversal of the read gradient G_R .

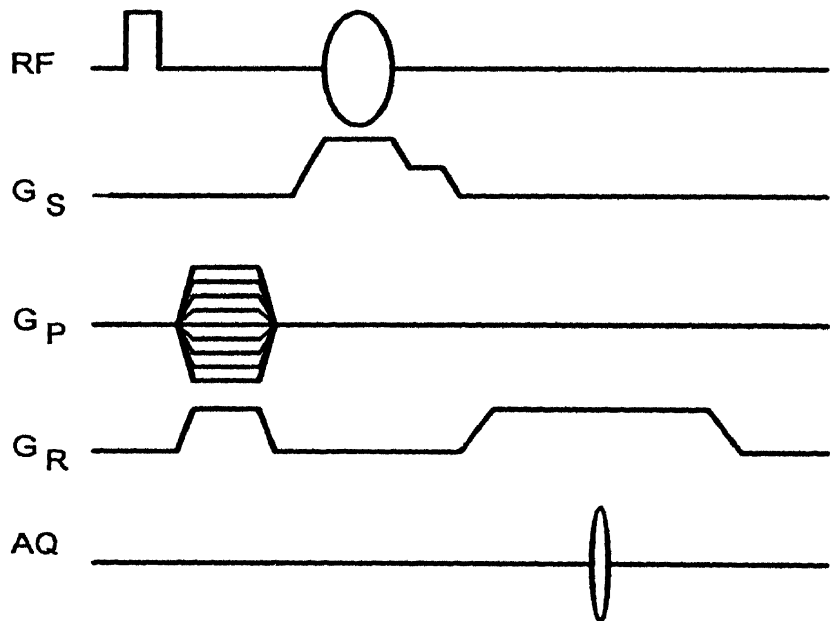


Fig. 2 Pulse sequence for a slice selective two-dimensional imaging experiment. The signal is acquired (*cf.* the AQ window) as a spin echo (a pair of back-to-back FID's). The echo is produced in the above protocol by a second rf pulse, the so-called refocusing pulse. Note that the read gradient is now not reversed, unlike the protocol of Fig. 1. The refocusing pulse is made frequency selective by shaping its envelope; selection of a desired plane is accomplished by issuing a slice selection gradient G_S during the shaped refocusing pulse.

sequences') for these two procedures are sketched above (Figs. 1 and 2).

One of the constant concerns in MR imaging is to optimize experiment performance in order to reduce measurement time and improve image contrast and resolution. One universal expedient in performance improvement is to register not 'single' FID's but instead to engineer the generation of echoes⁴ - which are pairs of back-to-back FID's and therefore double the signal intensity measured. Uniquely in MR imaging, it is possible to control the contrast in the image - *ie*, the variation of signal intensity across the picture elements ('pixels') - by a judicious choice of experimental conditions, invoking any or a combination of parameters: *eg*. nuclear isotope, chemical shifts, spin density, T_1 ,

T_2 , diffusion or flow. While MR image resolution is at best comparable to what can be achieved in optical microscopy¹, it is the superlative contrast capability that is the strength of the modality.

Indeed, contrast may be produced in MR images by employing anything from the simplest to the

¹This would not be possible but for the fact that MR imaging is physically not based on a scattering phenomenon (although it may be modelled mathematically as one), but is rather a resonance phenomenon. MR image resolution is limited by gradient strength, signal-to-noise ratio and by molecular self-diffusion over the time scale of the encoding and read-out process. Were it a scattering principle at work, the resolution would of course rather be limited by the wavelength of the radiation employed for rf-based work, this would correspond to something of the order of a metre!

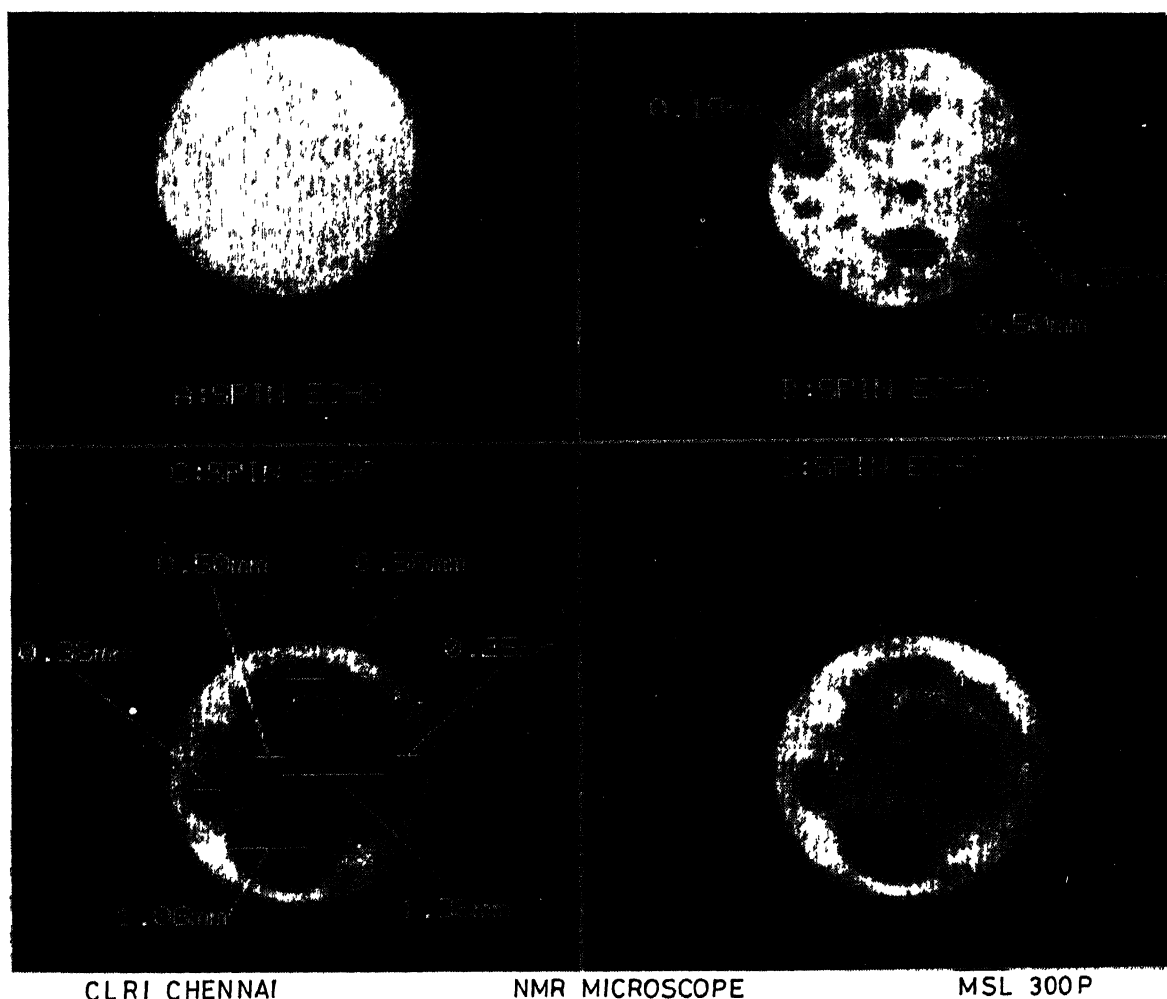


Fig. 3 Spin echo images of three gel samples namely sample A with no voids, sample B with a few voids and sample C with many voids, obtained on an 8.9 cm Bruker MSL300P system with an actively shielded microimaging probehead, employing a 5 mm ^1H RF insert; field of view FOV=8mm; number of scans NS=16; slice thickness=3 mm; spectral width SW=50 kHz. Image resolution: $63\mu \times 63\mu \times 3000\mu$. Echo time, TE is 7.05 ms and repetition time TR is 1020 ms. Note the visualization of pores with typical void sizes covering a decade (ranging from 0.19 mm to 1.38 mm). Voidage visualization in the spin echo images is in line with expectations from sample preparation.

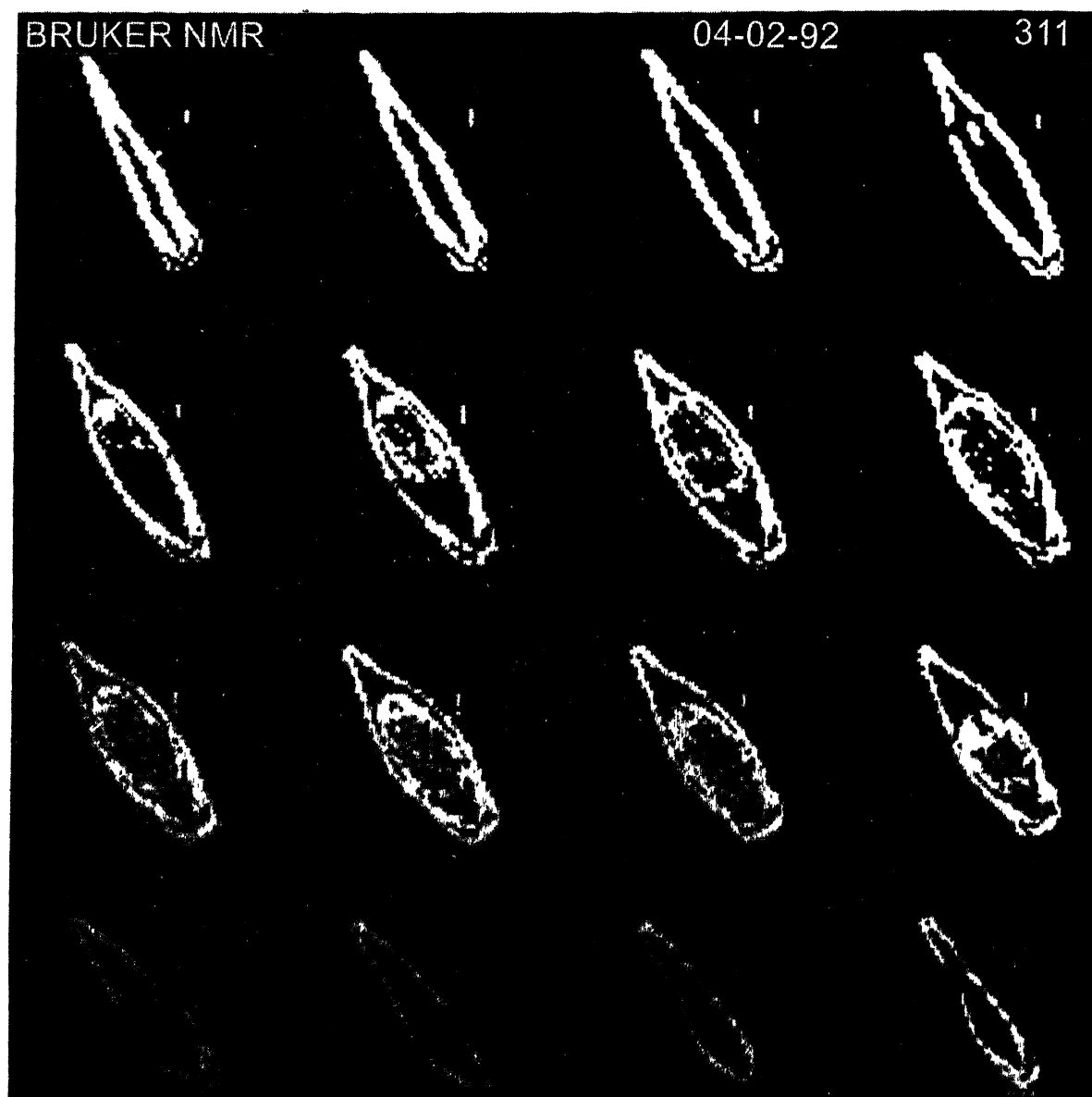


Fig. 4 Multi-slice images of *Avarai* (Tamil, a country bean).

most sophisticated magnetization manipulation strategies that are known - and constantly invented - in high resolution MR spectroscopy or relaxometry, for example by replacing the first RF pulse of the sequence by a suitable spin manipulation. It should thus come as no surprise that gradient echo images generate in principle a different contrast as compared to spin echo images.

From the point of view of measurement time, it is also quite clear that a 3D imaging protocol is far more time-consuming than a multi-slice 2D imaging protocol. Time savings in the multi-slice mode are typically at least a factor of 4, if not much greater when employing special tricks of interleaving or multiple-slice selective imaging (Figs. 3&4).

While the majority of imaging experiments have traditionally dealt with water protons or fat protons, which have been especially important in the biomedical context, the emphasis is now shifting to imaging more complex species with multiple resonances, as well as to imaging spins other than protons. This trend is bound to gather momentum with the increasing applications of MR imaging and microscopy to materials, as well as to a broad range of *in vivo* situations. We have developed a series of experiments that specifically address these issues related to imaging specific species — even if they be minor components — in a multi-component system.

In particular, we would like to mention three

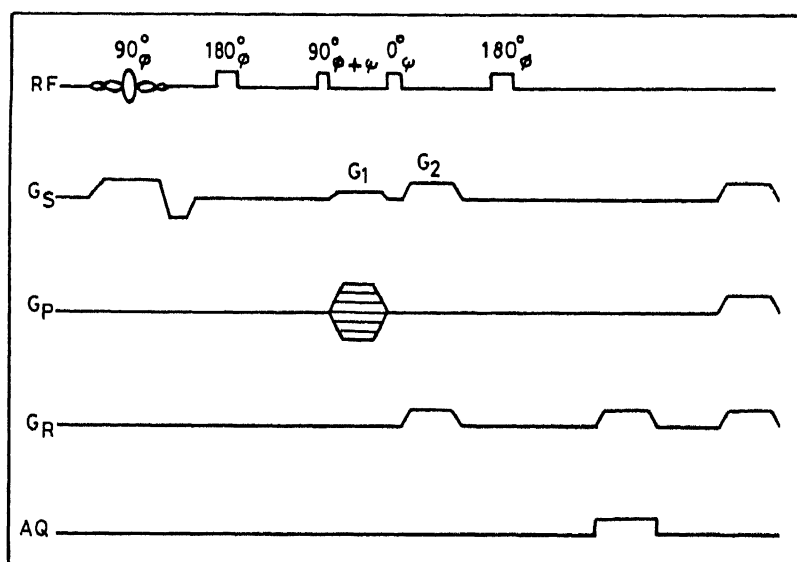


Fig 5 MUSQ Pulse sequence for 2D multiple quantum-single quantum imaging, combining multiple quantum phase encoding with single quantum frequency encoding, resulting in line scans in k -space. Coherence pathway selection is also made under gradient control.

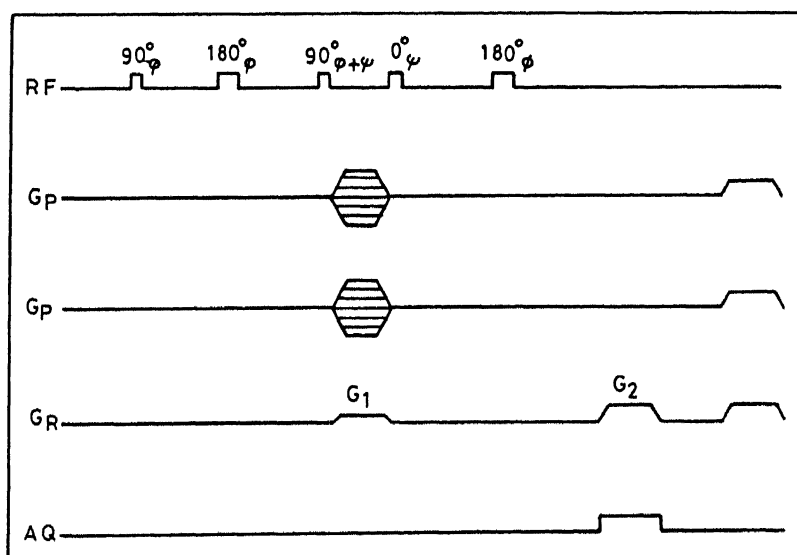


Fig. 6 MUUSIQ Pulse sequence for 3D multiple quantum-single quantum imaging, combining two multiple quantum phase encodings with a single quantum frequency encoding, resulting in line scans in k -space. Coherence pathway selection is also made under gradient control.

developments that we have originated: two- and three-dimensional multiple quantum imaging⁵, three- and four-dimensional multiple quantum spatial-spectral imaging, and an efficient new mixing sequence for imaging low γ spins⁶. The multiple quantum (MQ) experiments rely on the excitation and phase encoding of transitions — or coherences — of higher order than the normal,

directly observable single quantum signals which correspond to transverse magnetization. The MQ experiments involve a change in the magnetic quantum number of the spin system by more than one unit and offer a set of advantages against some trade-off in sensitivity. In particular, they allow molecule specificity, distinctive contrast, and resolution enhancement per unit applied field

gradient. n -quantum coherences are n times as sensitive to gradients as single quantum coherences. Their molecule specificity arises from the fact that in fluid-like phases, they may be created only when intramolecular, through-bond scalar couplings are present among the spins, corresponding to the bonding in the molecule

We have shown⁵ that in fluid-like phases it is possible to combine the advantages of multiple quantum phase encoding with single quantum frequency encoding, minimizing the experiment time in spite of the fact that MQC's are not directly observable¹ We have extended our MQ imaging experiments further to include imaging of ^2H , which has spin 1 and a quadrupole moment that

permits excitation of double quantum coherences (DQC) in partially ordered phases, such as in composites and lyotropic systems. Note that for such low γ situations, the effective amplification of γ that MQC's provide is extremely useful, because gradient amplitudes that would otherwise be required could be prohibitive! (For a given spatial resolution, the deuteron for example requires gradients that are a factor 6.5 larger than protons require, because of the ratio of their γ 's! On the other hand, DQC's of ^2H require only 3.25 times larger gradients.) It may also be noted further that in ordered phases where the spin-1 quadrupolar interaction dominates (or dipolar interaction, for an isolated pair of spins-1/2), there is a further factor that works strongly in favour of DQC imaging: in such systems, DQC does not, to first order, sense the interaction that broadens the corresponding SQC. There is thus a line narrowing effect at work here, in addition to gradient amplification: both of

¹In other words, we are back to scanning lines in reciprocal coordinate space, the so-called k space, instead of the point scan procedure that one would be 'condemned to' without frequency encoding

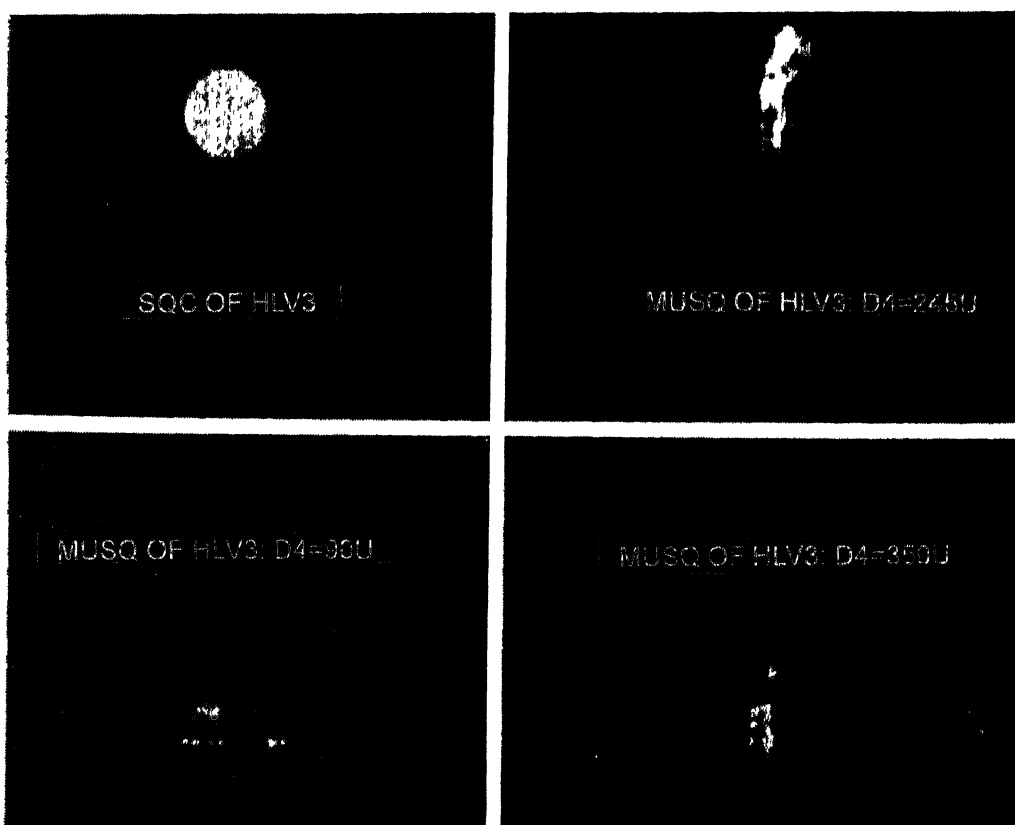


Fig. 7 Deuterium images of sample (HLV3) in lyotropic phase. Double quantum - single quantum MUSQ images of hexagonal phase were obtained with a 5 mm ^2H - ^1H insert. FOV=24 mm; NS=8, slice thickness=6 mm; SW=50 kHz; echo time: 8.17 ms, repetition time: 1000 ms; image resolution: $188 \mu \times 188 \mu \times 6000 \mu$. Double quantum MUSQ images obtained with different preparation times (D4), set at 245 μ s, 90 μ s and 350 μ s respectively correspond to double quantum creation being orientationally non-selective, or selective to parallel and perpendicular components respectively.

which contribute handsomely to image resolution enhancement per unit applied gradient. In operation, these multiple quantum imaging sequences must provide for the rephasing of anti-phase magnetization that is created in the first instance by the reconversion pulse that follows MQ phase encoding: else frequency encoding would result in very low profile intensity owing to cancellation effects (Figs. 5, 6 & 7).

The direct imaging of low γ species is fraught with more than one problem: to the classical problem of sensitivity is added the requirement of large gradient amplitudes to achieve reasonable image resolution. It is therefore doubly attractive to resort to proton detection schemes to indirectly image such low γ spins. For the indirect (*ie*, proton detected) imaging of low γ spins such as ^{13}C , we have also developed a family of pulse sequences (which we call PRAWN) that permits sensitivity enhancement by selective heteronuclear J cross-polarization, achieved with average rf field amplitudes of the order of the coupling constant, and thus with reduced power deposition in the sample compared to conventional cross-polarization methods used heretofore. This is clearly a vital consideration for *in vivo* applications, but is often a concern for materials investigations as well. Our method employs Pulsed Rotating frame tRAnsfer sequences with WInDows, in contrast to conventional methods that employ continuous trains of phase modulated pulses. Typical rf duty cycles in the train amount to 10% - 20%. The basic PRAWN sequence already exhibits a useful absolute Hartmann-Hahn mismatch tolerance range (*ie*, sensitivity of cross-polarization efficiency to the deviation from equality of the nutation frequencies of the two hetero-spins), which may be viewed as the corresponding tolerance range for cw JCP at the same average rf field amplitude, scaled up by the inverse of the PRAWN rf duty cycle. Our new sequences – including an explicitly mismatch compensated version with a refocusing pulse pair in phase quadrature sandwiched into the basic PRAWN train – have proved very efficient as front-ends tagged on to a suitable spin echo (or gradient echo) imaging protocol, *eg.* in imaging carbohydrate transport and metabolization in plants *in vivo*, as well as in visualization of elastomeric polymers in polymer blends⁷⁻⁹.

Note incidentally that the generation of gradient

echoes in such coupled spin systems works only when the development of anti-phase magnetization is avoided by issuing a refocusing pulse on the hetero-spin at the mid-point of the echo time TE.^k We have also developed an Echo Planar Imaging (EPI) version of the sequence, relying on a train of refocusing pulses on the hetero-spin at the zero crossings of the alternating read gradient to avoid the problem of multiple images corresponding to the spin coupled multiplet. Typical 2D imaging times – when dealing with ^{13}C enriched species – range from 1 to 10 hours with the spin echo version of the sequence applied to real life systems such as plants and polymer blends, while the EPI version has permitted imaging a phantom in 800 ms¹⁰.

It should be mentioned on the other hand that in contrast to such liquid-like systems, the imaging of rigid solids is quite another proposition. Here, the natural linewidth of the MR signal is so large (typically 100-1000 times the linewidths exhibited by liquids) as to require application of field gradients that are effectively two or three orders of magnitude larger than with liquid-like systems. One approach to this is again multiple quantum imaging, albeit with point scans in k -space. It seems more universally applicable however to employ the steady gradients that are naturally available in the fringe of a large superconducting magnet, and perform imaging by moving the sample across a suitable constant-field-plane in the fringe field. This method is known as STRAY Field Imaging (STRAFI)^{11,12} and takes recourse to the projection reconstruction strategy^l instead of the normal Fourier imaging approach that is used otherwise.

^kThis is in interesting contrast with the situation that prevails for MQ imaging, where the coupling must be given play to allow rephasing of anti-phase magnetization for successful frequency encoding.

^lThe projection reconstruction approach relies on producing a series of projections or profiles of the object as a function of the relative orientation of the object with respect to the gradient. The set of projections is then used in a reconstruction algorithm such as filtered back projection to retrieve the image. The principal motivation to employ this approach is to circumvent the requirement of fast gradient switching that is involved in methods related to Fourier imaging. Note that phase memory is retained during the sequence of gradient switchings that are required in this mode of operation. Such fast switching becomes technically more and more demanding the shorter the T_2 – as is the case with solids, and with electron spins.

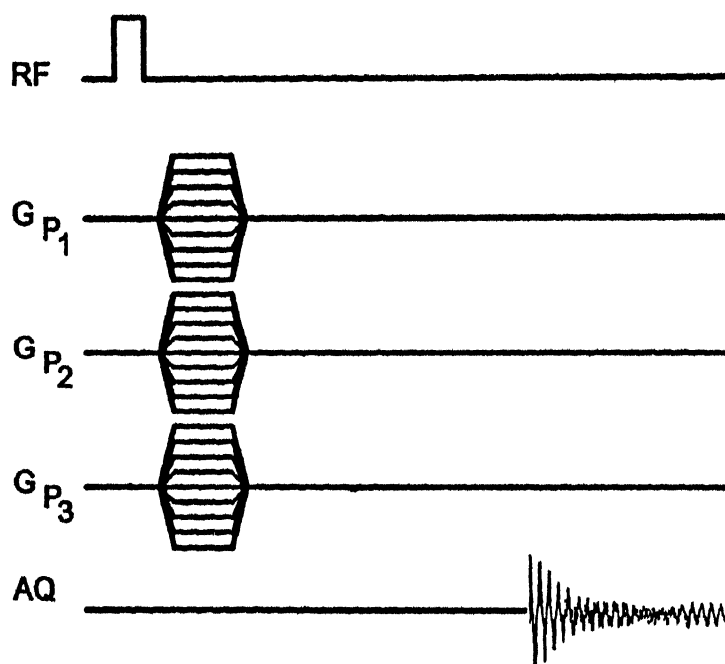


Fig. 8 Pulse sequence for chemical shift imaging, here shown in a four-dimensional (3 spatial, 1 spectral) version

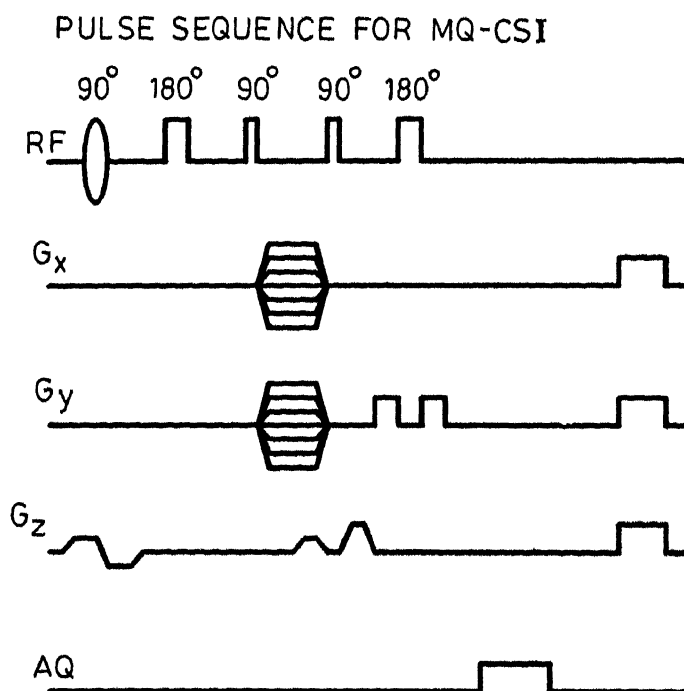


Fig. 9 MQ-CSI pulse sequence.

Volume Localized Spectroscopy

Field gradients enable the labelling of magnetization with spatial co-ordinates - in terms of frequencies and / or phases^m. Having attached

^mUnder suitable conditions, the encoding may be in terms of amplitudes as well.

these labels, the interest arises in then picking up high resolution spectral information that reflects the normal molecular level of detail: in other words, spectral information from specified volume elements ('voxels') of an object.

Two approaches are possible in principle to achieve this objective. On the one hand, it is

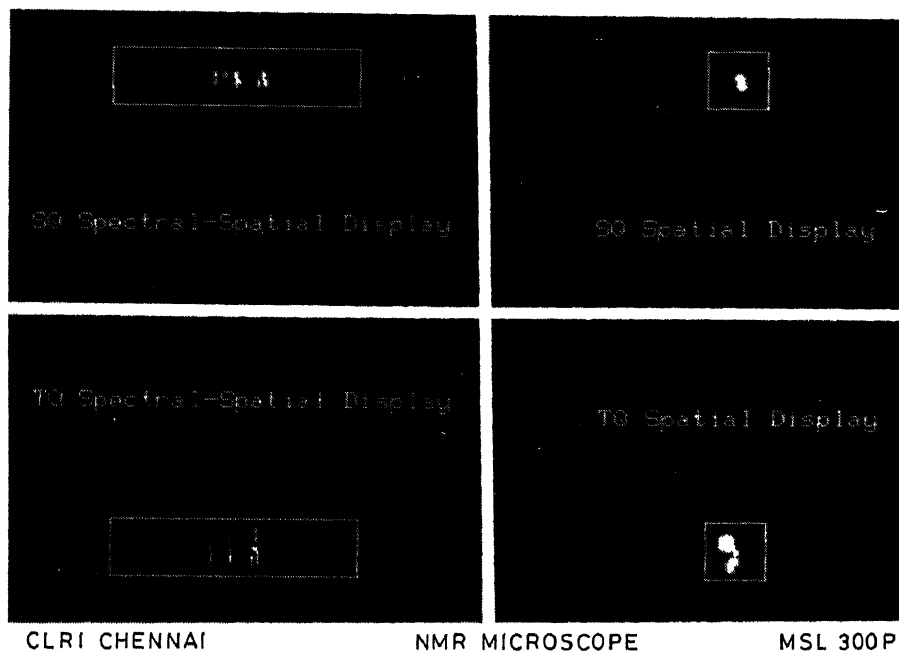


Fig 10 MQ-CS image of ethanol-propanol phantom, obtained with a 10mm proton insert on a Bruker MSL300P system with actively shielded gradients.

Bottom right. triple quantum image, SW=1778 Hz; FOV=24 mm, slice thickness=3 mm, NS=4, *left* spectral-spatial display

Top right Single quantum image, SW=1724 Hz, FOV=24 mm, slice thickness=3 mm, NS=4, *left* spectral-spatial display

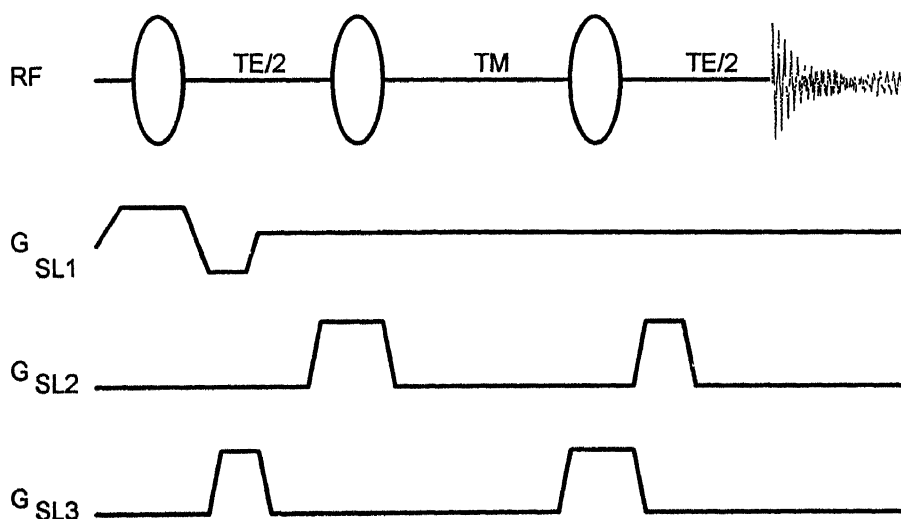


Fig 11 Pulse sequence for volume selective spectroscopy (called VOSY or STEAM).

possible to obtain the spectral information from all voxels in an object simultaneously, by the methods of chemical shift imaging (CSI). The principal difference of this mode of operation with respect to standard imaging protocols is that MR signals are now read out in the absence of gradients, following phase encoding in as many dimensions as

requiredⁿ. It is often the case that spectral read-out must be preceded by suppression of the strong

ⁿNote that frequencies, phases and amplitudes of precessing magnetization are all that MR ever senses. These quantities could be made to reflect either (intrinsic) molecular properties or spatially encoded information—or both—by a judicious choice of experimental conditions.

response from 'uninteresting' molecular species, eg. water *in vivo*. It is also possible to make the spectral readout a function of more than one time variable, for example by interposing further spatially unencoded spectral evolution period/s before actually reading out the signal. The resulting dataset could thus be multi-dimensional both in spatial and spectral domains. A typical experiment protocol to produce CSI is shown in Fig. 8.

The principal shortcomings of such CSI experiments relate to the fact that a frequency encode in real time has been substituted by a phase encode in 'virtual' time, thus leading to considerably longer data acquisition times. As a compromise, one reduces the number of phase encode steps. This has the consequence however that the 'point spread function' exhibits sinc wiggles owing to truncated sampling of reciprocal co-ordinate space (k -space), resulting in 'leakage' of spectral information between voxels^o. It is also often the case that one is not necessarily interested in spectral information from every single voxel in the object in the first place.

We have developed a multiple quantum spectral-spatial imaging procedure, MQ-CSI¹³. Owing to the properties of MQC's (apparently higher γ), k -space is sampled more effectively, generating a 'cleaner' point spread function, thereby reducing contamination (or inter-voxel leakage). Note further that the resulting spectrum corresponds to a

multiple quantum filtered experiment, which is very efficient at suppression of undesired information such as solvent peaks (Figs 9 & 10).

At the other extreme, one may however choose to seek spectral information from a single selected voxel of interest, reducing inter-voxel leakage considerably, but losing the multiplex time advantage of the multi-voxel CSI procedure¹⁴⁻¹⁸. This procedure may in turn be developed into a multi-dimensional spectroscopy protocol. A highly successful pulse sequence to produce volume localized single voxel spectra is shown in Fig. 11.

We have also produced two-dimensional zero quantum spectra *in vivo*, operating in the volume localized STEAM environment, by systematic incrementation of TM and optimization of the pulse flip angles in the sequence to suppress coherence transfer to passive spins. This strategy goes a long way in enabling unambiguous identification of metabolites in low field MRS, thanks to the correlation information that is picked up between coupled spins.

Measurement of Transport

Effects of field gradients on spin echoes had been noted very early on in the development of time domain (pulsed) MR. It was realized that spin echoes could suffer attenuation by molecular self-diffusion occurring in the presence of field gradients. Echoes essentially refocus the dephasing of transverse magnetization under the influence of 'inhomogeneous interactions', by enabling a time reversal of the motion of the magnetization. Such refocusing is incomplete to the extent that

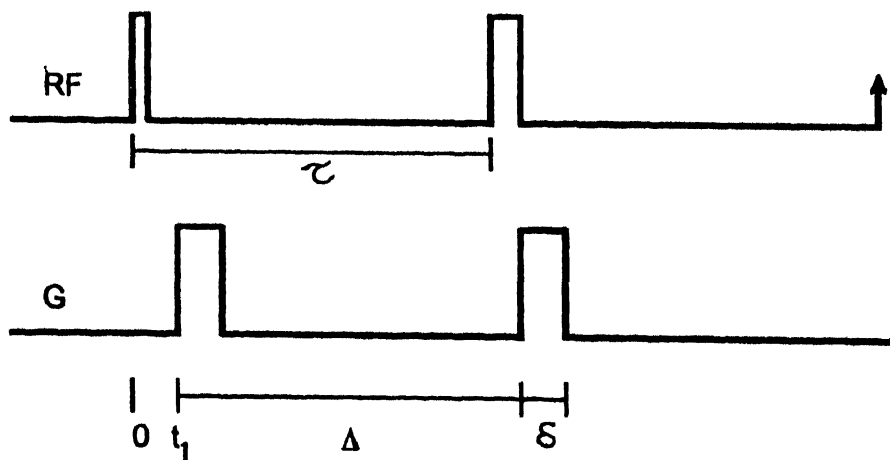


Fig 12 Pulse sequence for PGSE experiment to measure molecular self-diffusion coefficients.

^oRecall that truncated sampling in time domain, for instance, is equivalent to multiplying the signal with a gate, corresponding to convolution of the spectrum with the sampling (or 'sinc') function in frequency domain.

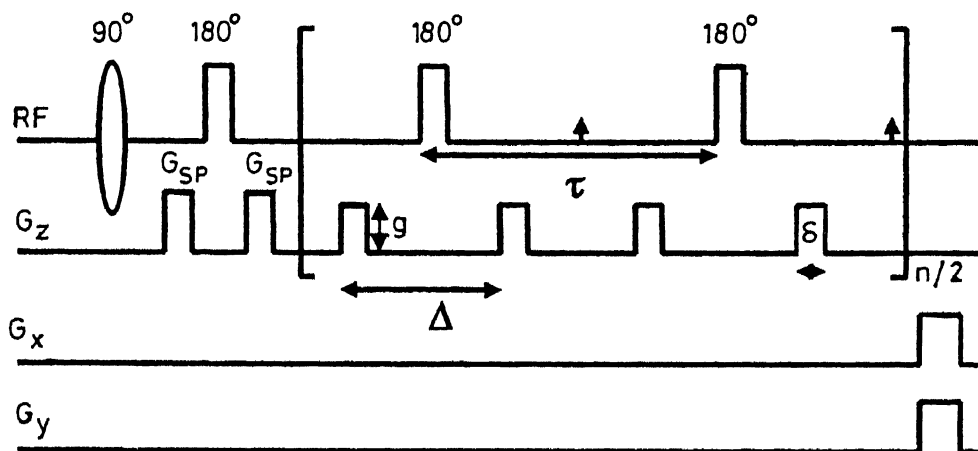


Fig 13 FAMOUS pulse sequence for 1D chemical shift selective echo spectra with diffusion encoding. The shaped 90° pulse has optimum bandwidth to select the desired chemical shift in single-/multi-component systems. A refocusing π pulse with symmetric spoiler gradients G_{sp} is applied thereafter; this counteracts dephasing during the shaped pulse. The π pulse train then commences after the appropriate rephasing delay.

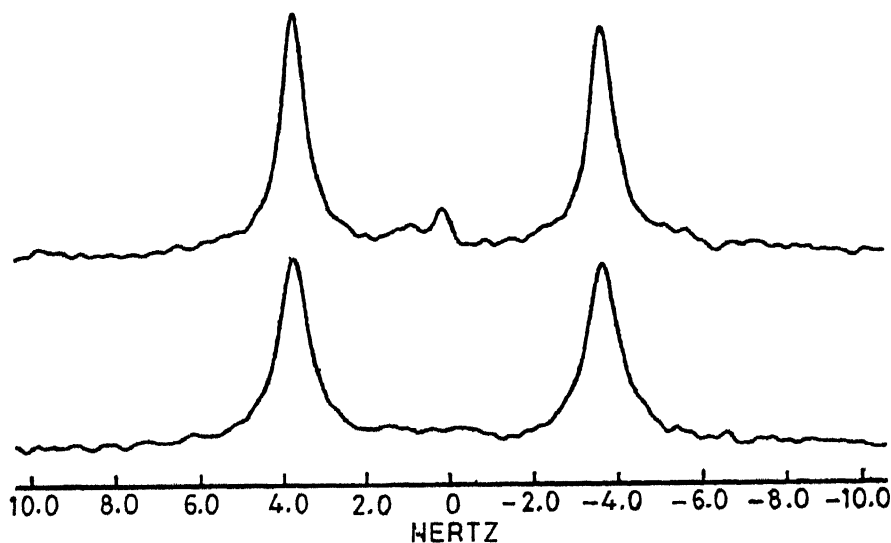


Fig. 14 Application of shift selective FAMOUS on the methyl doublet of 100 mM *N*-acetylalanine in D_2O . The lower trace displays the diffusion encoded J spectrum with linewidth of 0.81 Hz for the peaks at $\pm J/2$ Hz. The upper trace displays the spectrum obtained without diffusion encoding with linewidth of 0.67 Hz for the peaks at $\pm J/2$ Hz. The additional broadening of 0.14 Hz resulting from the application of the diffusion gradient of 16.33 G cm^{-1} corresponds to a diffusion coefficient of $0.46 \times 10^{-5} cm^2 s^{-1}$. Shift selection was achieved with a Gaussian pulse of duration 20 ms, issued at the methyl chemical shift. (Adapted from S Sendhil Velan and N Chandrakumar)²².

irreversible processes - including diffusion of molecules from one region of the magnetic field to another with a different intensity - occur during the spin echo pulse sequence. In general, a coherent flow of spins¹⁹ results in a phase shift of the echo, while a random diffusion process results in a distribution of phase shifts whose ensemble average translates to an amplitude attenuation.

These findings were elaborated to the pulsed gradient spin echo (PGSE) method²⁰ for measuring flow and molecular self-diffusion coefficients. Note incidentally that MR is the only technique available that genuinely measures self-diffusion coefficients (Fig. 12).

Typically, the measurement of molecular self-diffusion coefficients D by MR involves the

systematic repetition of such a pulse sequence as the one shown above, varying the gradient pulse amplitudes and/or durations. One captures the echo top and recovers the diffusion coefficient from an analysis of its attenuation as a function of gradient pulse area. The measurement is typically a 30 - 60 minute affair and works excellently for single component systems. One of the merits of the pulsed gradient protocol is that it weights equally all diffusion events that occur during the interval Δ .

For multi-component systems on the other hand, with the possibility of different D values for different components, a modified PGSE approach is required since the echo top masks by and large the chemical shift differences among components and therefore precludes their individual identification. One possibility to recover this information would be to capture not the echo top but instead the entire second half (or even the whole) of the echo and perform a Fourier transformation to generate a series of spectra as a function of gradient pulse areas²¹. The typical measurement time remains 30-60 minutes for a multi-component system.

We have developed²² a fast two-shot method to handle such situations, by generating a series of spin echoes, capturing the series of echo tops and Fourier transforming them to generate a spectrum which is essentially governed by couplings. Upon repeating this procedure - once without gradient pulses and a second time with (or in principle with two different values of gradient pulse areas) - one generates a pair of spectra, the difference of the linewidths in which quantitatively encode D . (For non-Lorentzian lineshapes, a deconvolution procedure is required to extract the relevant information.) The basic measurement procedure lasts a couple of seconds. For multi-component systems, the procedure may be repeated with shift selective excitation (Figs. 13 & 14)

It may be noted that successful schemes have been developed also for diffusion- or flow-weighted imaging.

Prospects

From this brief overview of spatially resolved MR,

it is clear that the technique offers exciting vistas of applications of relevance to plant sciences, biomedicine and industry, while constantly renewing itself and widening its scope and finesse. Indeed, applications appear to be open-ended^P. As regards hardware, on the one hand wider bore magnets with active gradient shielding are becoming available at higher fields for materials and animal investigations (excluding clinical low field MRI, currently upto 50 cm bore at 4.7T and 20 cm bore at 9.4 T), while on the other hand, higher frequencies are also becoming available for small sample MR microscopy (currently upto 800 MHz at 18.8 T) The game is thus wide open for a stream of imaginative and fruitful exploitations of the technique. A recent analysis suggests, further, that the traditional electromagnetic coil detector of MR is after all likely to be better behaved than the cantilever detector of the emerging technique of Magnetic Resonance Force Microscopy^{23,24}, with respect to strong coupling and chaotic behaviour at smaller and smaller detector and sample dimensions.

Acknowledgments

It is a pleasure to acknowledge the contributions of Dr S Sendhil Velan, Dr V Visalakshi, Mr K Victor Babu and Mr S Venkata Raman to various aspects of the work described in the foregoing. The stimulation and close co-operation in the group of Professor R Kimmich (Universität Ulm, FRG) and the interaction with the groups of Professor P G Morris and Dr R Bowtell (University of Nottingham, UK) in the field of ¹³C imaging is gratefully acknowledged, as also the interest and stimulation of Hindustan Lever Research, ICI R&T and Unilever plc. The author thanks CSIR and DRDO for research funding and DFG for financial support during the collaboration at Ulm.

This contribution, as indeed the INSA Symposium of which it was a part, is dedicated in honor of Professor E M Purcell.

^P Except for bulk metals!

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MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY IN BIOMEDICINE

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The basic principles of NMR are maximally exploited in a very important contemporary medical modality for *in vivo* soft tissue diagnosis, namely, Magnetic Resonance Imaging (MRI). An overview is made of the cardinal aspects of the MR physics governing the various signal encoding methodologies and the signal intensity contrast mechanisms, including MR angiography of flowing spins, effects of diffusion and perfusion on MR images, and image contrast enhancement with exogenous paramagnetic agents. Applications of a host of methodologies to anatomical and functional (fMRI) imaging of living tissues are reviewed. An account is also given of the single- and multi-voxel localisation techniques for doing *in vivo* clinical spectroscopy (MRS) of tissue metabolites and their application potential in diagnostic medicine. Recent work in functional MR spectroscopy (fMRI) is also briefly reviewed.

Key Words : MRI; Functional Imaging; *In Vivo* ^1H and ^{31}P MRS; Spectroscopic Imaging; Functional MRS

1 Introduction

The phenomenon of NMR, the progenitor of Magnetic Resonance Imaging (MRI), was discovered over fifty years ago¹⁻³ and has since been an important spectroscopic discipline. It is common knowledge for the informed general scientist that NMR spectroscopic signatures such as the chemical shifts and coupling constants, and the kinetic parameters of the spin system such as the spin-lattice relaxation time (T_1), the spin-spin relaxation time (T_2), and the spin diffusion coefficient (D), are some of the most useful quantities for investigating matter from the molecular perspective^{4,5}. However, the adaptation of NMR principles and methodologies for noninvasively studying the living human being has been a relatively recent, and spectacular, technological leap following developments in signal encoding techniques and signal intensity contrast mechanisms⁶⁻⁸. Today, MRI is a pre-eminent soft tissue diagnostic modality for many neurological, musculoskeletal, cardiac and other pathologies^{9,10}.

separated water-filled capillaries was reported in 1973¹¹. Four years later, a demonstration of the first human tissue images using NMR was published¹². It is of historical interest to recall that Lauterbur's experiment¹¹ used narrow-band c.w. NMR in a d.c. magnetic field gradient to acquire projections of the signal along different directions which were later 'reconstructed' into an image, and that several other early strategies employed rapid a.c. magnetic field gradients to define 'sensitive lines' or 'sensitive points' which were rastered spatially to acquire image data directly^{13,14}. Useful as they were in early research prototypes insofar as they required less ambitious data rates and digital electronics, they were unacceptable for practical medical imaging. The current imaging methods fall overwhelmingly in the category of 'Fourier imaging' and exploit properties of Fourier transforms as shown by Ernst *et al.*^{5,15,16}. Our present discussion is therefore confined to pulsed gradient Fourier imaging techniques. By the mid-1980's, following intense activities in laboratory level technology design¹⁷, system modelling¹⁸, etc., full fledged commercial systems started appearing on the world market, and imaging applications of

The first crude NMR imaging on two spatially

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NMR became a standard part of radiologic practice. Also during this phase, the naming of this modality was reconsidered. To eliminate the layman's perceived 'hazardous' connotation in the original christening of NMR, the first letter was dropped and 'MR' imaging/spectroscopy gained currency as the standard *in vivo* terminology.

Some additional points need to be made before evaluating the significant advances in this field. Although sustained efforts have been made since the late 80's to perfect an MR 'microscope' capable of resolving volume elements smaller than $100\text{ }\mu\text{m}$ ^{3,19-22} and despite the fact that even MR images of rather large single cells²³ and single neurons²⁴ have been published, the natural limitations on the *in vivo* resolution imposed by physics have so far precluded MR imaging of common intact mammalian cells. Fortunately, this limitation is offset by the fact that the physical principles of MR are far more versatile and can lead to additional valuable information on tissue function and metabolism. Indeed, the full complement of normal diagnostic MRI (for mapping soft tissue morphology), functional MRI or fMRI (for studying tissue function), MR angiography or MRA (for flow depiction and quantitation), and MR spectroscopy or MRS (for studying tissue biochemistry *in vivo*) will together provide noninvasive insights at the cellular and molecular levels into normal physiology and pathophysiology of the living tissue.

Accordingly, our focus in this review will be on how MR signals from living tissue yield *functional* and *metabolic* information in addition to the more conventional images suitable for *morphological* diagnosis. Core concepts such as image formation and image contrast shall form the centrepiece of our theme, and adjunct application methodologies based on blood flow and water diffusion will be reviewed. Illustrative examples will be cited, if somewhat selectively, from a host of reports on *in vivo* tissue pathologies (including appropriate animal models), our choice being often based on work where the methodology/information content is either representative or novel. In this choice, however, the author should plead guilty to a certain inevitable element of subjectivity.

2 Principles of Tissue Magnetisation, Resonance and Image Formation

The physics of MRI is based on the inherent

magnetism possessed by the hydrogen nucleus, or the proton (¹H). Due to its continual subparticular (quark level) reorientation²⁵, each proton behaves as a spinning charge which sets up an associated magnetic field. The proton therefore has a 'spin' magnetic moment, and is a microscopic magnet. Soft biological tissues, in particular, contain hydrogen in the form of water and fat, and are therefore modelled as ensembles of tiny magnets in random orientation. By placing the patient in a powerful and uniform static magnetic field B_0 whose preselected and fixed value is of the order of a Tesla ($\cong 1\text{ T}$) along the tunnel axis (z) of the MR imager, the tissue of interest is magnetised. In the frequently used vector model²⁶, the tissue proton 'magnets' perform a rapid, phase-coherent wobbling motion which is identified as the familiar Larmor precession, with a frequency ω and with a resultant magnetisation M_z along the direction of B_0 . For values of B_0 in the Tesla range, ω will fall in the megahertz (MHz) range.

Application of an RF pulse of amplitude B_1 , which may be seen as a burst of RF power over a certain bandwidth tuned to the centre frequency ω , will then cause the protons of the magnetised tissue to 'resonate'. That is, due to excitation by the tuned RF pulse, the M_z magnetisation is torqued (or tipped away) from the direction of B_0 to produce a precessing component M_{xy} in the transverse plane. The degree to which M_z is tipped away is called the 'flip angle', α , and it increases with the duration of the applied RF pulse and its amplitude.

The precessing M_{xy} produces a small electrical signal - the MR signal - in a tuned RF receiver coil surrounding the tissue region of interest (ROI). The RF receiver circuit of the imager picks up and amplifies the voltage of the MR signal. This voltage is a quickly decaying oscillation in time, called the free induction decay (FID). In basic NMR, the frequency equivalent (ω) of the energy that a proton absorbs at resonance and re-emits as the FID signal is determined by the exact B_0 value that it experiences, namely,

$$\omega = \gamma |B_0|, \quad \dots (1)$$

where γ is the gyromagnetic ratio, and is 42.6 MHz T^{-1} for the proton.

Being a waveform, the FID corresponding to each resonating species (water, fat, etc.) is a composite of three important properties, viz., the signal amplitude, frequency (f) and phase (ϕ).

While the amplitude is a measure of the number of resonating nuclei (proton density), f and ϕ may be exploited together to 'code' the resonance signal at each time domain point

It shall be seen that it is the manipulation of the FID that gives us the time domain data to create MR images. One could either use the FID directly, or create refocussed echoes of the FID to create images. Also, we briefly recall here the three important spin relaxation processes that influence the observable MR signal: T_1 characterises the rate at which nuclear spins restore equilibrium by re-aligning with B_0 after they have been perturbed initially by an RF pulse; T_2 characterises the more intrinsic mechanism of the precessing M_{xy} components dephasing each other, and T_2^* is a related time-constant characterising a foreshortening of T_2 due to magnetic field inhomogeneities and susceptibility differences between different tissues ($T_2^* < T_2$).

The fundamental principle of image formation is that the MR signal frequency, eq. (1), can be made

to depend linearly on the spatial location of the group of nuclei producing the signal⁶⁻⁸ by applying moderate (upto 20 mT m⁻¹) linear gradients. For the imaging experiment, eq. (1) is modified to

$$\omega_i = \gamma(B_0 + \mathbf{G} \cdot \mathbf{r}_i) \quad \dots (2)$$

where ω_i is the frequency of the proton at position $\mathbf{r}_i$ and $\mathbf{G}$ is the vector representing the gradient amplitude and direction. It is therefore easy to see how a 1-D image projection is made. The signal is acquired in the presence of a field gradient and the intensity is plotted vs. the frequency¹¹, with the frequency axis being 'coded' for, i.e., be proportionately equivalent to, the spatial position. Images in two or three dimensions are made by an extension of this coding concept, using additional magnetic field gradients along the y - and z -axes, and recognising that a precessing spin experiencing a gradient pulse of duration Δt along either of these axes will accumulate an additional phase, e.g.,

$$\phi_y = \gamma G_y \Delta t \quad (3)$$

Various RF pulse sequences (Fig 1) can be used

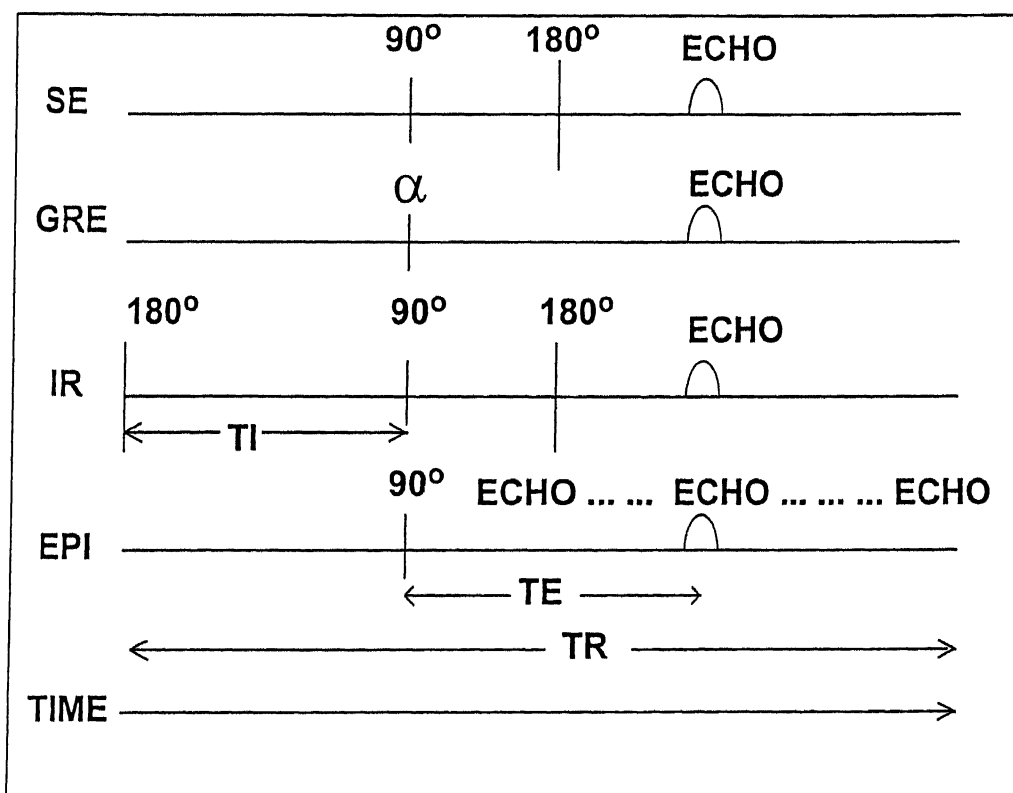


Fig 1 Most frequently used MRI pulse sequences. In each sequence, the timings of the RF pulses (but not the gradient pulses) are shown, and signal data are acquired as an echo. SE spin echo, GRE gradient recalled echo (α , the pulse flip angle, is usually much less than 90°), IR inversion recovery, EPI echo planar imaging. TR repetition time between two successive sequences. TE echo time, or time between RF excitation and echo formation, TI Time after inverting the initial magnetisation.

for resonating and refocussing the tissue magnetisation to produce images. At various times during these RF sequences, magnetic field gradients are switched on according to a protocol that enables reconstruction of the required image at the end of the data collection. Many repeated acquisitions are made using the same pulse sequence to obtain all the information needed to reconstruct the image, with different gradient phase-encode pulses applied on each pass.

The most frequently used MRI sequence; the spin echo (SE) sequence in which the 90° RF pulse is followed by a 180° pulse, is shown at the top line of Fig.1. This sequence, being a close analogue of that used in NMR spectroscopy to measure $T_2^{4,5}$, can lead to T_2 -weighted images. The timing parameters TE and TR of the SE sequence can be varied to achieve contrast sensitivity. The signal intensity, S , may be given by^{9,10}

$$S = CM_z [1 - \exp\{-(TR - TE/2)/T_1\} + \exp(-TR/T_2)] \times \exp(-TE/T_2) \quad (4)$$

In eq. (4), C is a constant related to the amplification of the MRI receiver, and M_z reflects the proton number density. For short echo times (TE), eq (4) simplifies to

$$S = CM_z [1 - \exp(-TR/T_1)] \exp(-TE/T_2) \quad (5)$$

Typical proton T_1 and T_2 values for various human tissues at 1 T and at 1.5 T are given in Table I. Whereas the T_2 's are relatively uninfluenced by the B_0 value, T_1 's are generally shorter at lower B_0 values. One observes from Table I that simple, undiscriminated proton density images can be too 'even-handed' in that they show only $\sim 10\%$ signal intensity variation over the whole range of tissues. However, by choosing appropriate TR , TE parameters in the SE sequence, the T_1 and T_2 values can be exploited for tissue identification and, subsequently, delineation

of a pathology (e.g., tumour). It is this unique ability of MRI to introduce dynamic levels of soft tissue contrast for probing both tissue density and microscopic biochemical environment that renders it such an important modality in diagnostic radiology.

To understand the gradient recalled echo (GRE) sequence appearing in the second line of Fig 1, we see that an initial, small angle ($< 90^\circ$) RF excitation pulse (α -pulse), applied at $t=0$, flips the precessing tissue magnetisation into the transverse (xy) plane. If we now apply a uniform gradient field G_x for a time t , then according to eq. (3) the spins will accumulate a phase

$$\phi_{initial} + \int_0^t \gamma G_x X dt.$$

Whenever the second term, i.e., the time integral of G_x , becomes zero the transverse magnetisation regains its original phase *regardless of its position*. If all the spins are in phase at some time t_1 , they will regain this phase at a later time t_2 forming an

echo, provided $\int_{t_1}^{t_2} G_x dt = 0$. This means that a

simple reversal of the gradient G_x produces the gradient echo. Likewise, several periodic reversals of G_x will produce a train of echoes, which is the principle involved in EPI (Fig. 1). The EPI technique shall be discussed later.

It is important to note that the gradient echo amplitude depends on T_2^* (rather than T_2 as for the SE) because all spatially fixed spin-dephasing processes such as magnetic susceptibility variations will not be refocussed into the echo by the gradient reversal. Because GRE sequences do not employ 180° RF pulses, they can be executed with much shorter TR values. This encourages the use of a small α ($< 90^\circ$) to prevent spin saturation and signal reduction. For these reasons, GRE was the original fast-scan MRI sequence, introduced under the acronym of FLASH (fast low-angle shot) by Frahm²⁷. On the third line of Fig. 1 is depicted the inversion recovery (IR) sequence which introduces contrast through inverting the initial tissue magnetisation by a 180° RF pulse and then allowing the various tissue protons (water, fat, blood) to recover for an interval TI (the inversion time) before applying a 90° pulse and obtaining a signal. Either an FID image or a spin-echo image is obtained. Tissues with slightly different values of

Table I
Typical Proton T_1 and T_2 Values of Living Tissue at 1.0 T and 1.5 T

Tissue	T_1 (ms)		T_2 (ms)	
	1.0 T	1.5 T	1.0 T	1.5 T
Grey Matter	810	920	100	100
White Matter	680	780	90	90
Fat	240	260	85	80
Muscle	730	860	45	50
Cerebrospinal Fluid	2500	3000	1400	1420
Blood	525	600	260	260

T_1 , e.g., the grey matter and white matter of brain (Table I), become well contrasted in inversion-recovery images.

A very important application of the IR sequence is for *fat saturation* (or 'fatsat' as it is sometimes called). Fat has a T_1 of 200-250 ms at 1.5 T. If a value of 140-150 ms. is selected, the excitation pulse occurs when the fat magnetisation has no longitudinal (M_z) component and produces an image with no fat signal. Signals from other tissues have variable hyperintensities according to their T_1 values. These principles form the basis of the STIR (short T_1 inversion recovery) technique, which is often used by radiologists to diagnose a pathology in fat-rich regions (liver, spine, etc). Alternatively, a choice of long T_1 values allows the magnetisation from semi-solid tissues to return to equilibrium, and only the very long T_1 species such as fluids are nulled. This is the basis of the *fluid attenuation by IR* (called FLAIR) sequence²⁸ which can be pre-inserted in T_2 -weighted SE or fast SE sequences to delineate focal lesions while suppressing signals from fluids, e.g., cerebrospinal fluid (CSF).

The complete timing diagrams of MR imaging sequences, illustrated extensively in the literature^{9,10,35}, are customarily divided into three operational modules: slice selection, phase encoding and frequency encoding (or 'readout'). Each module occurs at a different time during the sequence, and each employs gradients along different axes so that spatially specific image detail can be obtained. There are no restrictions as to which gradient axis is used for which of the three options defined above, and this permits the radiologist a considerable latitude in defining the direction from which the image is viewed. Images in usual tomographic planes of the object, namely, axial, coronal, sagittal or oblique, are easily obtained by making the appropriate axis definitions for the various gradient pulses.

The slice selection module involves applying a gradient, G_z , simultaneously with the RF pulses, designed to excite the nuclear signal in only a narrow, well-defined RF band. In Fig. 2(a), selective excitation of an axial slice through the brain is illustrated. In the Fourier imaging

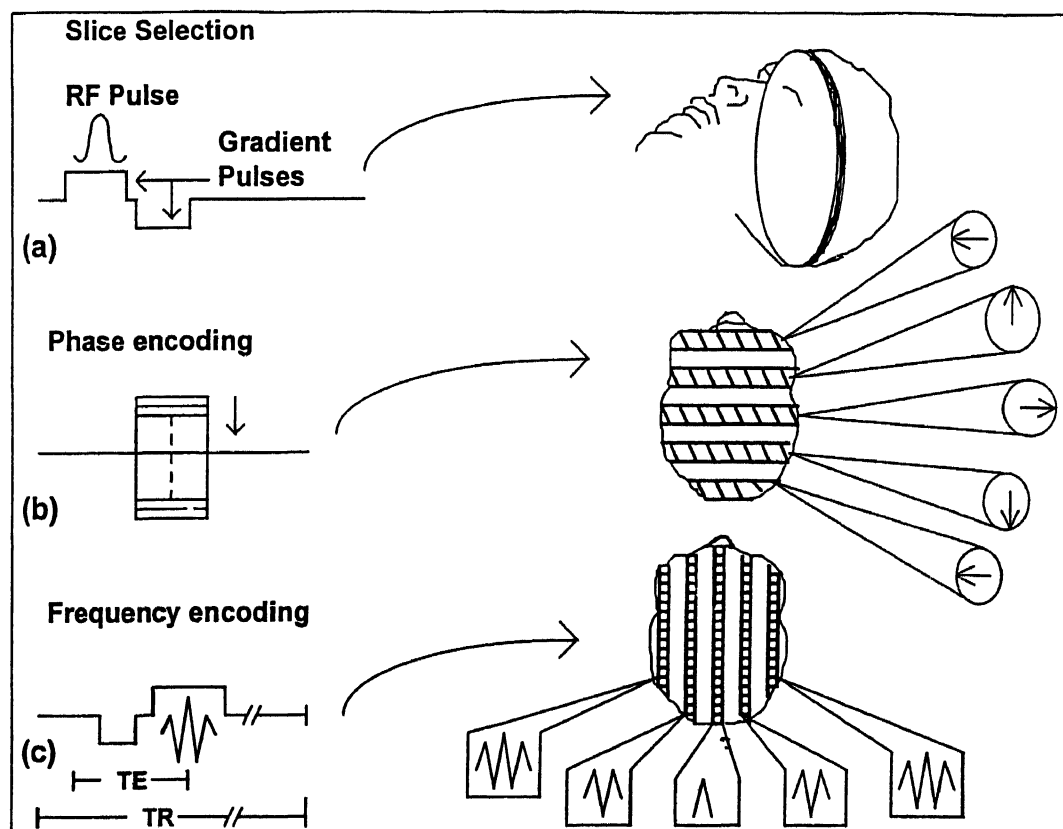


Fig. 2 Slice selection, phase encoding, and frequency encoding modules, shown with their respective magnetic field gradient pulses on the left. The effect of each module is illustrated on the right for selecting and coding an axial slice through the head

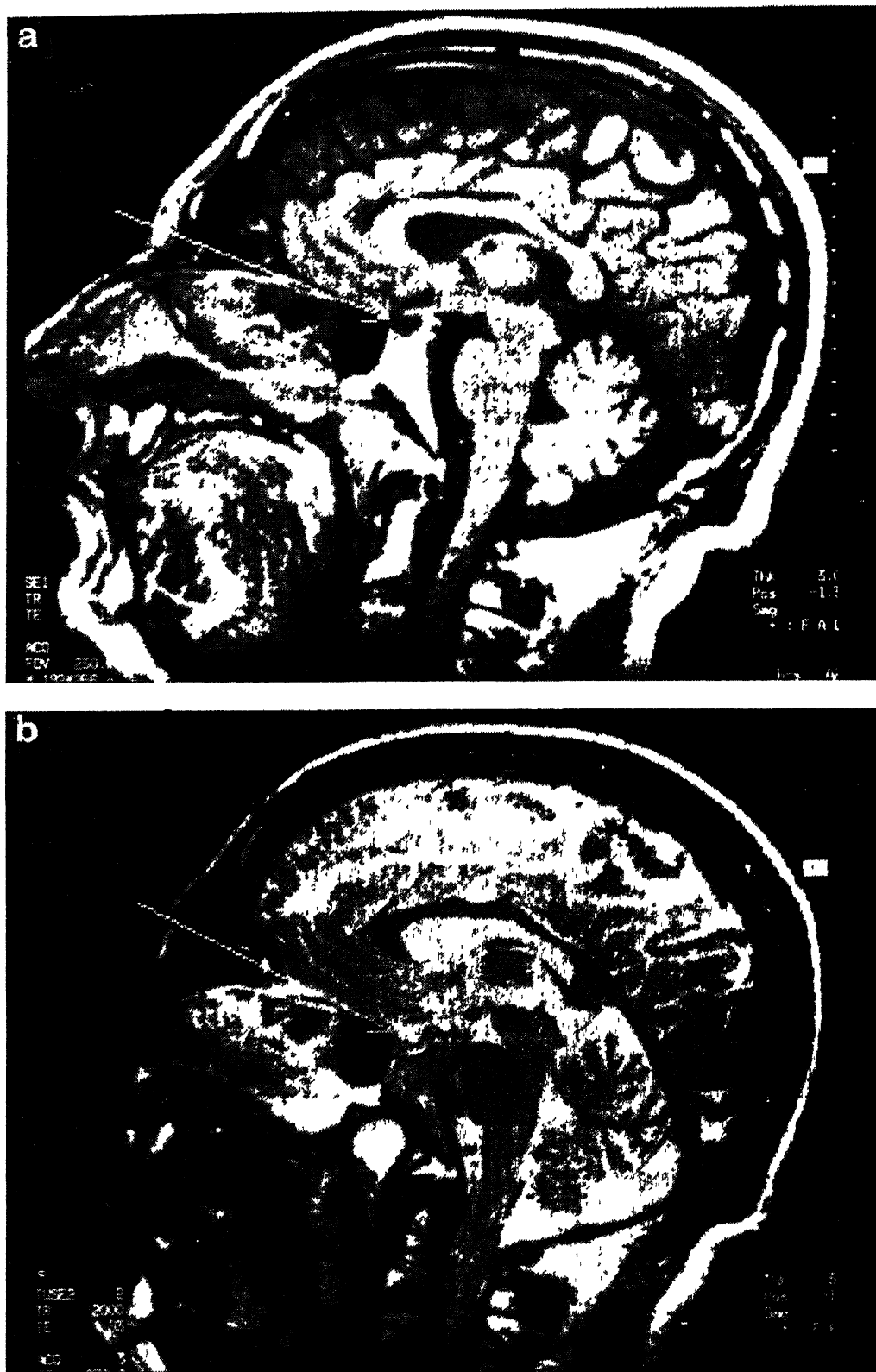


Fig 3 MRI of the mid-sagittal brain (a) T_1 weighted image, (b) T_2 weighted image. Contrast of the pituitary gland (arrow-marked) is much higher in the T_1 -weighted image.

formalism we shall outline below, the 2-D spatial distribution of the tissue protons, $A(x,y)$, is mapped by rendering their precession frequencies spatially dependent. The phase-encoding and frequency-encoding modules, illustrated in Figs. 2(b) and 2(c), respectively, are used to sample the spins in the two directions orthogonal to the direction of slice selection. Acting together, they partition the chosen slice into a grid of pixels so that a 2-D image of the selected slice can be reconstructed.

The phase-encoding gradient pulse, G_y , makes the phase of the transverse magnetisation different in each of the rows shown on the right hand side of Fig 2(b). Magnetisation from tissues within each row has the same phase. Frequency encoding is based on sampling the transverse magnetisation in the presence of G_x . This gradient gives transverse magnetisation a frequency that depends on the column in which it is located. The precession frequency is identical within any single column. The echo forms at time TE. After a time TR, the three procedures are repeated the same way, except that the G_y assumes a different amplitude.

By using eq. (3) the time-domain signal, $S(t_x)$, sampled in the presence of G_x applied for a time t_x may be seen to be¹⁸

$$S(t_x) = \int A(x,y) \exp[i\gamma \int_0^{t_x} G_x(t) x dt] dx dy \quad (6)$$

The other in-plane gradient, G_y , applied consecutively for a time t_y modulates the above signal to yield

$$S(t_x, t_y) = \int A(x,y) \exp[i\gamma \int_0^{t_x} G_x(t) x dt + i\gamma \int_0^{t_y} G_y(t) y dt] dx dy \quad (7)$$

It is seen straightaway that a double Fourier transform of eq. (7) yields $A(x,y)$, the required MR image.

The MR image is customarily displayed on a 2-D device such as a video screen, with the x - and y -co-ordinates of the various pixels representing anatomical position within the subject being scanned, and the brightness at each point

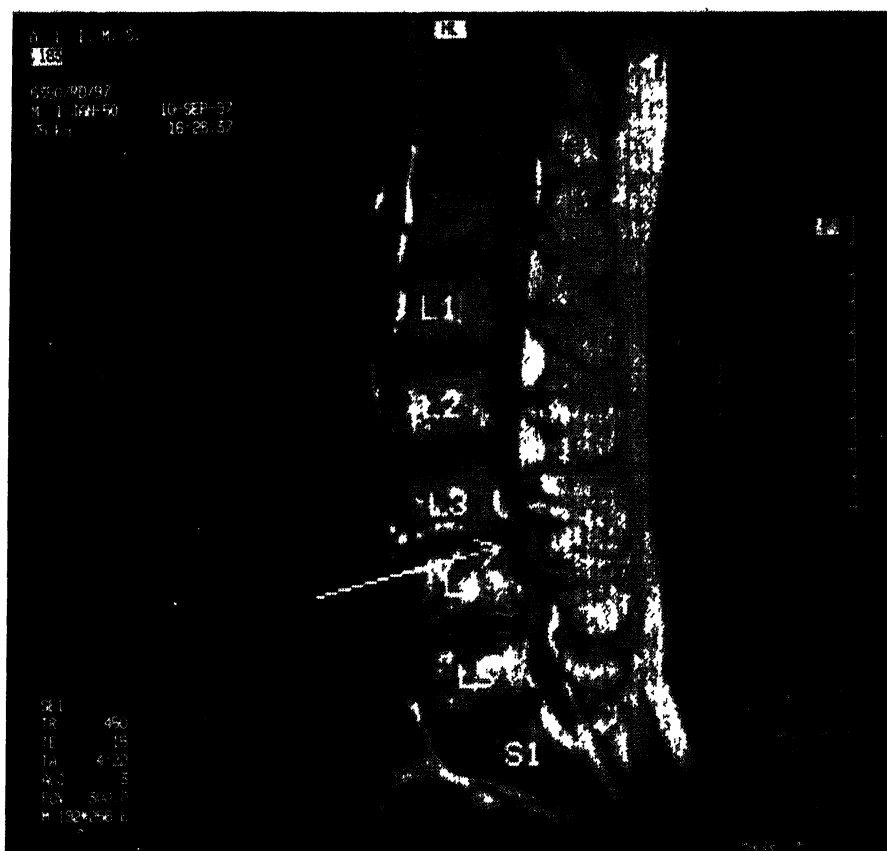


Fig 4 Sagittal section of T_1 -weighted MRI of lumbar spine, showing L1 to L5. Region of disk prolapse is shown by the arrow.

representing the MR signal intensity. The pixel intensities are usually stored as 32-bit words with a 16-bit dynamic range and displayed on either a 16-bit or an 8-bit greyscale monitor. Figs 3 and 4 are examples of the exquisite inherent contrast afforded by T_1 - and T_2 -weighted spin-echo images. In the T_1 -weighted mid-sagittal brain slice, Fig 3(a), the grey and white matter distinction is exemplary, clearly delineating the cerebral cortex, the lateral ventricle, the pons, the cerebellum and, in particular, the pituitary gland (marked by the arrow). The T_2 -weighted image of the identical slice, Fig 3(b), is seen to highlight white matter and CSF to the exclusion of other details, particularly the pituitary. In Fig. 4, a badly prolapsed disk in the L4 region (arrow-marked) typifies a radiological diagnosis using T_1 -weighting.

Perhaps the largest application area of T_2 -weighting is in clinical neuroimaging. In many cases MRI is the first examination to indicate that a central nervous system (CNS) disorder is predominantly or exclusively affecting the white matter²⁹. To cite some representative examples from our own recent work, the clinical utility of T_2 -weighted MRI was demonstrated in a somewhat rare brain neoplasm, namely, intracranial non-Hodgkin lymphoma³⁰. The effectiveness of T_2 -weighting was also demonstrated in a series of studies aimed at characterising very early stage lesions and cytotoxic edema accompanying experimental induction of brain neuron damage³¹⁻³³. T_2 -weighted imaging was also employed in an experimental (rat model) study of acoustical damage to the brain parenchyma³⁴.

To overcome any limitations in image details brought about by slice thickness (usually 2 mm or more), 3-D imaging techniques have also been described³⁵, where the slice selective pulses are eliminated and phase-encoding is used in two dimensions with the third being interrogated by a readout gradient.

The need for signal acquisition through repetitions of the pulse sequence for phase-encoding is one of the reasons why SE MRI is a relatively slow process. If a resolution of 128 lines is desired along the phase-encode direction, then an equal number of passes through the sequence would be required to complete the picture grid, or the raw data matrix in the time domain, which we shall call k -space.

3 The Concept of K-Space

It was realised early in the development of MRI³⁶⁻³⁸ that by means of the simple substitutions

$$k_x = \gamma \int_0^t G_x(t) dt \text{ and } k_y = \int_0^t G_y(t) dt \quad \dots (8)$$

the MR signal, eq (7), can be re-cast in terms of spatial frequencies. A more appropriate term than 'spatial frequency' would be 'spatial phase', since k_x and k_y actually express the phase 'advance' or 'retardation' the spins experience per unit tissue length in the x - and y -directions.

A transparent analogy with the familiar 'reciprocal space' concept in optics and crystallography now emerges. Just as in the diffraction limit in optics, it is the wavelength $\lambda = 2\pi/k$ that decides the spatial resolution achievable in MRI, and *not* the wavelength of the RF at the Larmor frequency. The spatial frequency axes k_x and k_y constitute the k -space grid of time domain or 'raw' data which is the Fourier inverse of the MR image. More exhaustive treatments of k -space concepts have been published^{39,40}.

The raw data or 'diffraction pattern' of the object is characteristically peaked around the k -space origin⁴¹, and when the time evolution of the gradients carries the sampling trajectory past this point, a sharp maximum in the MR signal, well known in the classical NMR literatures as an 'echo'⁴², is observed. To generate such an echo, either an additional refocussing (180°) RF pulse must be applied to the resonant spin magnetisation after the initial RF excitation (this generates the SE) or a gradient must be inverted to refocus the spins (this generates the GRE). Every MR image in frequency space, defined by TR , TE , flip angle, etc., is uniquely paired with its own k -space raw data set, meaning that the two can be interconverted by Fourier transformation. Contrast information becomes encoded in the central area (around $k=0$), while image spatial resolution is encoded in the outer peripheries of k -space. In clinical MR images, the k -space has its own maximal intensities near the origin.

In conventional protocols, e.g., SE, the k -space is stored by sampling n lines each of m data points. Each line is measured from left to right, this direction being determined by the sign of the G_x gradient. The distance of a horizontal line from the origin is determined by the sign, height and

duration of G_y . Data from a single line is called a 'view', or a 'profile'.

In a dual-echo SE experiment, Fig. 5, two images with different TE values but otherwise identical parameters are obtained. That is, in one dual-echo scan, two k -space data matrices are acquired, each of different TE . During the scan, data are acquired by alternatively measuring a view in the first echo k -space and then a view in the second echo k -space.

4 Faster K-Space Acquisition Techniques

One of the abiding concerns of MRI technology has been to reduce image acquisition times to more acceptable levels. Newer designs have optimised pulse sequences to enable the acquisition of a single line, or view, of k -space in only a few milliseconds, providing GRE images in a second or less. In SE imaging, a technique to reduce imaging time has been the RARE (Rapid Acquisition by Relaxation Enhancement) sequence, originally introduced by Hennig^{43,44}. In its generalised form it is also known as the turbo spin echo (TSE) protocol.

(i) Turbo Spin Echo (TSE)

A TSE protocol resembles multi-echo SE (Fig.5) in that it uses a train of 180° pulses. The difference, however, is that each echo is phase-encoded differently. This enables the measurement of several views following a single RF excitation of a slice, and all these views are mapped in the same k -space. The result is that only one (or a few) TSE scans (i.e., one or a few TR values) are needed to acquire the raw data for the image.

Consequently, this k -space is filled with views each of which belongs to a different TE. However, since image contrast is effectively determined around the origin of k -space (the low- k views), the echo time used to produce the $k=0$ view is taken as the 'effective' TE , and the sequence is planned such that other low- k views have roughly the same TE . Usually the effective TE is half way along the echo train. Typical echo trains are 200-300 ms. long and contain upto 30 echoes (30 is called the 'turbo factor').

The main advantage of TSE over its conventional counterpart is the very significant reduction in scan time. Image contrast is somewhat different, however. For example, fat appears

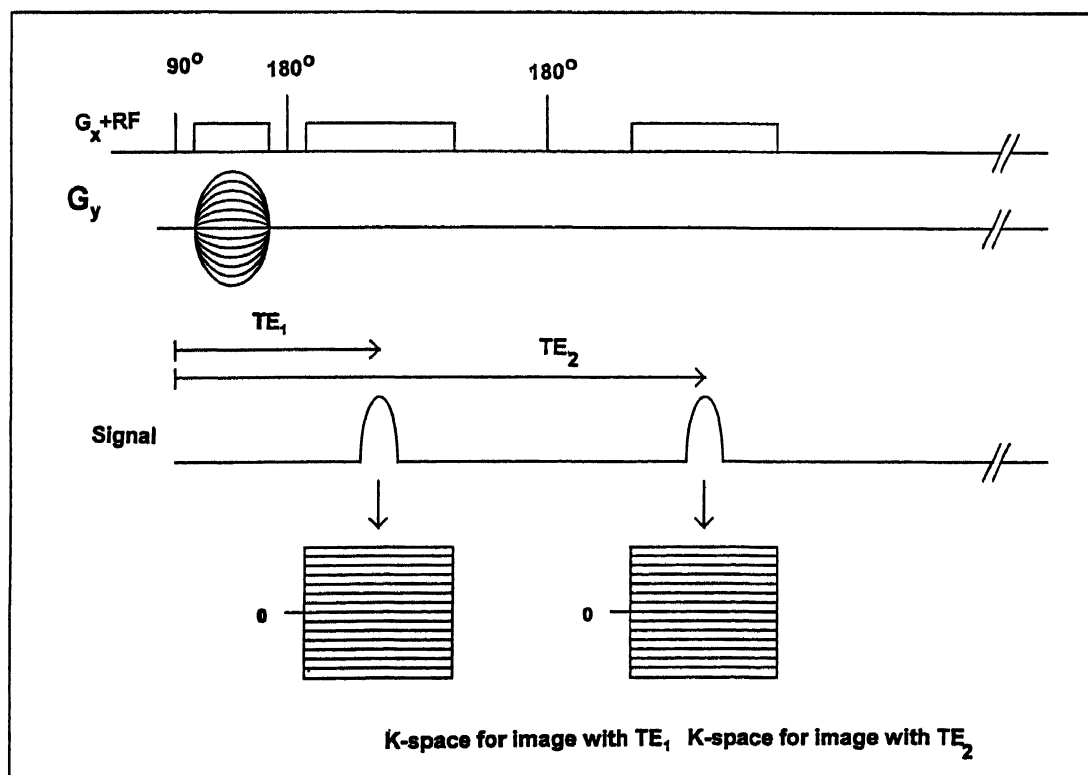


Fig 5 Pulse sequence and k -space data matrix showing a 'dual echo' SE protocol

brighter due to echo time, which still carries some T_1 -weighted contrast. Also, TSE is less sensitive to magnetic susceptibility effects so that, for example, hemorrhage with hemosiderin appears less dark and can be diagnosed. The larger RF power deposition in the patient will cause a practical lower limit to the echo spacing; in most cases, a spacing of 10-12 ms. can be attained.

(ii) Echo Planar Imaging (EPI)

EPI is a 'snapshot' strategy to map the multiple phase-encoded lines of k -space after only one RF excitation; in other words, it is a protocol wherein the complete data set required to reconstruct an image is acquired from the MR signal produced by a single RF excitation of the selected slice. This original concept⁴⁵, and its early real-time biomedical application⁴⁶, employed a continuous scan path, zigzagging rectilinearly through the entire k -space plane. This requires a clever manipulation of the encoding gradients whereby G_x is inverted after each line-scan while G_y is applied very briefly in 'blips' after completion of a line. Inversion of a gradient inverts the scan direction in k -space. G_x is preferably strong and rapidly switched to obtain very fast spin echoes (1/ms), while G_y is blipped as narrow pulses coinciding with the zero crossings of the G_x gradient. Programmed to act in synchrony, these gradients generate the fast zigzagging route through the k -plane. The MR signal is continually sampled during this k -space traverse, and the method is therefore ultrafast and efficient.

EPI may be implemented in either the SE or the GRE version. If a 180° pulse is applied between the initial excitation pulse and the start of sampling, the sequence becomes the SE EPI.

EPI is somewhat limited by the fact that S/N is relatively poor. Each line must be digitised within a very short time, since all lines have to be scanned before the T_2 -decay of the spins following excitation. Short acquisition time for each line requires a large receiver bandwidth and, consequently, noise is higher. Spatial resolution is also usually limited.

Several other variations of EPI have been implemented in recent years, all essentially differing only in the way k -space is scanned in one pass following a single RF excitation of the tissue, e.g., ultrafast spiral scanning⁴⁷, segmented or interleaved k -space trajectories⁴⁸, and interleaved

gradient-echo planar imaging, IGEPI⁴⁹. The universal feature in all these methods is the traversal of an area (plane), rather than a line, of k -space for each excitation.

In an obvious extension of the above concepts to the third dimension, Mansfield has recently introduced the technique of Echo Volumes Imaging, EVI^{50,51}.

5 Flowing Spins and MR Angiography (MRA)

While flowing tissue (e.g., blood, CSF) normally produces severe artefacts in an image, MRA turns this to advantage to make it the primary source of signal intensity in the image⁵². MRA enables visualisation of normal, laminar blood flow within the vascular system and its disruption due to any pathology such as a thrombus (blood clot). Also, MRI can be of particular use in evaluating vessel patency. Present biomedical practice is to use the so-called 'bright-blood' technique, that is, signal from flowing protons is enhanced relative to the stationary protons through the pulse sequence and measurement parameters. The bright blood depicts arteriovenous malformations, aneurysms, stenoses and other diseases familiar to the physician. Bright blood MRA methodology can be divided into time-of-flight (TOF) and phase contrast (PC) techniques.

MRA essentially envisages both arterial and venous flow through a certain volume of interest. To avoid an ambiguous diagnosis between these two types of flow, spatial presaturation pulses are applied to saturate out the unwanted blood signal prior to its entry into the imaging volume (Fig. 6). At the other limit, presaturation pulses can also be used in 'black blood' techniques where both arterial and venous blood signals are saturated out, and the vessels appear dark relative to the surrounding tissue.

A common problem with MRA procedures is an exaggerated sensitivity to blood vessel stenosis. The stenotic region disrupts the laminar flow both in the area of, and distal to, the stenosis, causing signal loss from the imaged vessel. This can lead to an extremely variable appearance of the vascular lumina through which blood circulates. As the laminar flow is restored in the vessel, bright signal is seen again.

(i) Time-of-Flight (TOF)

TOF techniques are very effective and speedy for obtaining MRA images. A single measurement

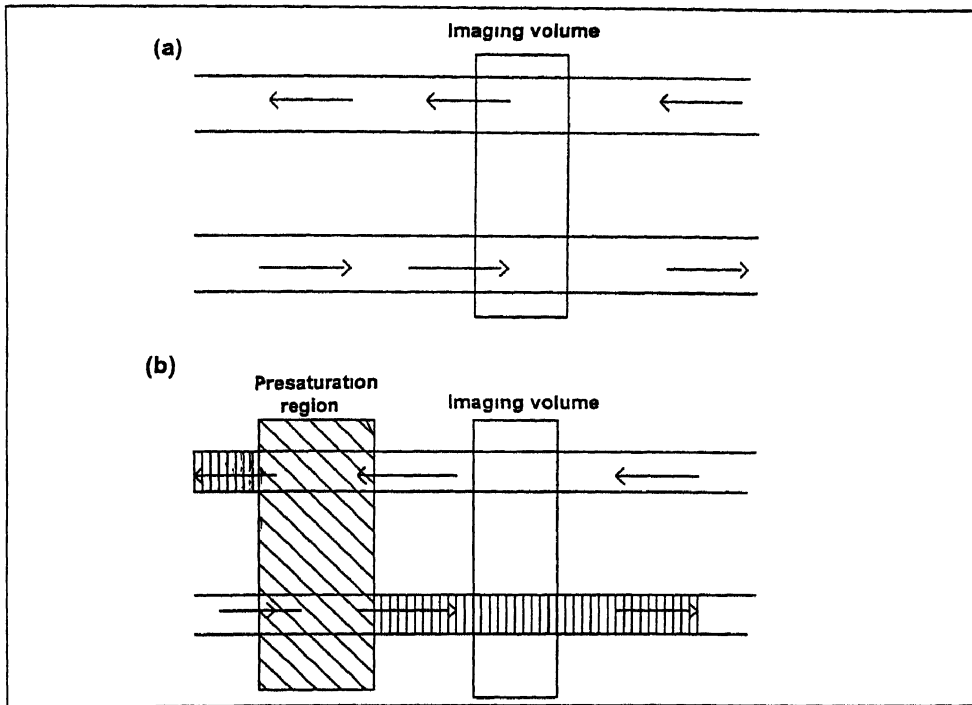


Fig 6 MR angiography (a) Without a presaturating RF pulse, flowing blood entering the VOI is imaged regardless of flow direction, (b) RF pulse presaturates flowing blood prior to its entering the VOI, suppressing the signal (Adapted from Ref 35, p 112)

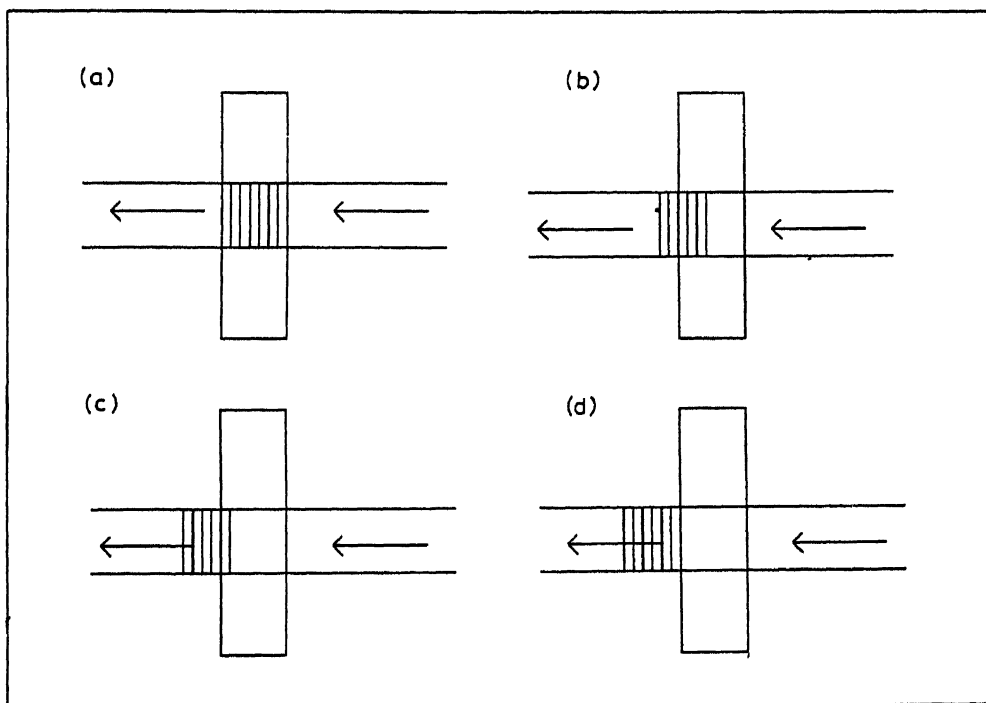


Fig. 7 TOF effect. VOI receives a series of RF pulses during data collection. (a) Flowing blood (striped box) gets excited by the first RF pulse, (b) During TR, the excited blood moves; (c) During second TR period, the flowing blood has moved again, (d) At the end of the third TR, the initial blood volume will be outside VOI and will not contribute to signal (Adapted from Ref. 35, p 113)

is performed, with the stationary tissue signal suppressed relative to flow. A TR much shorter than the tissue T_1 values and a moderate flip angle (α) are used. While the stationary tissue experiences every RF excitation pulse, the flowing tissue does not. The imaged blood volume will be at a different location at the time of each excitation pulse due to its flow during TR (Fig. 7). Due to the 'wash-in' or influx of fresh spins, signal from the blood volume is brightest at the entry point of the slice because it has not been hit by any excitation pulses. As the blood volume travels through the slice, it loses signal as it becomes saturated by RF pulses. If the flow direction is perpendicular to the observed slice (i.e., through-plane flow) and the volume of excitation is small, then the volume of blood exits the slice before it is completely saturated and significant blood signal will light up the entire slice. The degree of saturation of the flowing blood depends upon the slice thickness, TR, α and flow velocity.

TOF may be run in 2D-sequential or 3D-volume acquisition modes. The former is preferred for imaging slow vascular flow, such as venous flow, or to limit saturation from in-plane flow. It may also be used to acquire slices during the interval of a patient's 'breath-hold' to limit abdominal vessel misregistration and respiratory motional artefacts. 3D-TOF provides the best in-plane resolution and minimises vessel misregistration.

TOF techniques suffer from incomplete suppression of stationary tissue signals due to their shorter relaxation times relative to blood (Table I). Magnetisation transfer techniques^{53,54} are often incorporated for additional suppression of the stationary tissue to enhance small vessel detectability. 3D-TOF also suffers from saturated out blood signal due to the large number of RF excitation pulses, and is therefore suitable only for vessels with fast flow, e.g., medium and large arteries. One strategy to overcome this saturation problem, namely TONE (tilted optimised nonsaturating excitation), uses RF pulses with spatially dependent flip angles⁵⁵. A low flip angle is used for the entry slice where inflowing blood is unsaturated, and the flip angle is increased linearly across the slab to compensate for the decreased magnetisation of the flowing blood as it traverses the volume, becoming progressively more saturated. Excellent images of the abdominal aorta have been demonstrated by this method⁵⁵.

(ii) Phase Contrast (PC)

When a spin flows in a magnetic field gradient it accumulates an additional phase relative to that of a stationary spin. The amount of this accumulated phase depends on the direction and velocity of the spins relative to the applied gradient, and on the gradient amplitude and duration. Considered quantitatively, the stationary spin at X_0 experiencing a gradient G_x for a time Δt will have a rotation angle $\phi_0 = \gamma G_x X_0 \Delta t$ (see eq. 3). The flowing spin starting at X_0 and moving with a velocity V_x will have moved to a position $X_0 + V_x \Delta t$ after Δt and will have experienced different (and increasing) magnetic fields as it moved up the gradient. This results in the accumulation of an extra phase angle $\phi_t = 0.5 \gamma G_x V_x (\Delta t)^2$. This extra phase shift is directly proportional to the flow velocity and the applied gradient strength but rapidly increases with the square of the time spent in the gradient.

For constant velocity flow, this extra phase shift ϕ_t will add a constant offset to the phase encodings at each phase-encoding step and will lead to the corresponding image location having an additional phase shift after the Fourier transformation. This is the basis of PC MRA techniques, since sites without moving spins will have only random (noise) phase shifts while blood-flow sites sensitised by the PC method will show specific and coherent ϕ_t 's. By this method flow volume quantitation is possible by summing the ϕ_t 's over the area of the blood vessel. Many life-system processes and nonideal responses also lead to signal phase shifts and, therefore, to specific image artefacts. For this reason, most PC procedures use subtraction of flow-desensitized from flow-sensitized acquisitions to remove the effects of these artefactual phase processes.

Owing to their directional sensitization, PC methods enable separation of the flow into individual directions⁵⁶. They also have better background suppression compared with TOF. However, PC suffers from two problems. One is a significantly longer scan time. Four separate acquisitions are required to measure all three flow components, resulting in a fourfold increase in scan time compared with a TOF measurement with similar acquisition parameters. The other problem is that prior knowledge of the maximum flow velocity is necessary to ensure that the proper

sensitizing gradient pulse is used. Slow flow velocities will not be visualised if a large amplitude gradient pulse is used, while excessively fast flowing spins will be misregistered as slow-flowing, which is essentially an 'aliasing' artefact.

6 Diffusion and Perfusion Imaging

(i) Diffusion Weighted Imaging (DWI)

As distinct from the locally coherent directional motion which is usually understood as flow, we now examine the other kind of microscopic motion of water protons, namely, randomly directed Brownian motion or diffusion, as a potential contrast mechanism. For motion in one direction, the mean translational distance, $\langle L \rangle$, that a water molecule would diffuse in time t is given by the so-called Einstein equation

$$\langle L \rangle = (2 D t)^{1/2} \quad \dots (9)$$

where D is the diffusion coefficient of water.

The effect of diffusion on the MR images may be understood from a SE sequence in which a pair of bipolar field gradient pulses is included (Fig. 8). The purpose of these gradient pulses is to

magnetically 'phase tag' the diffusing spins, in much the same way a radiotracer is used to tag a molecule in the more classical diffusion studies in physiology⁵⁷. In Fig. 8, following Stejskal and Tanner⁵⁸, we denote the gradient pulse amplitude and duration by G and δ , respectively, and the interval between gradient pulses by Δ .

For two spins diffusing past each other, the first gradient pulse induces a phase shift of the spin transverse magnetisation depending on the spin position a_1 (spin tagging). Likewise, the second gradient pulse will produce a phase shift which depends on the spin position a_2 at this time (spin untagging). Due to the 180° RF pulse, the nett phase shift will be

$$\phi = (\gamma G \delta) (a_2 - a_1). \quad \dots (10)$$

It is immediately seen that for 'static' spins, $a_1 = a_2$ so that the bipolar gradient pair has no effect on phase. For diffusing spins, however, there is a nett phase change, which depends on the spin history during the interval Δ between the gradients that affects the transverse magnetisation.

We now consider the macroscopic magnetisation arising from the vector sum of the individual

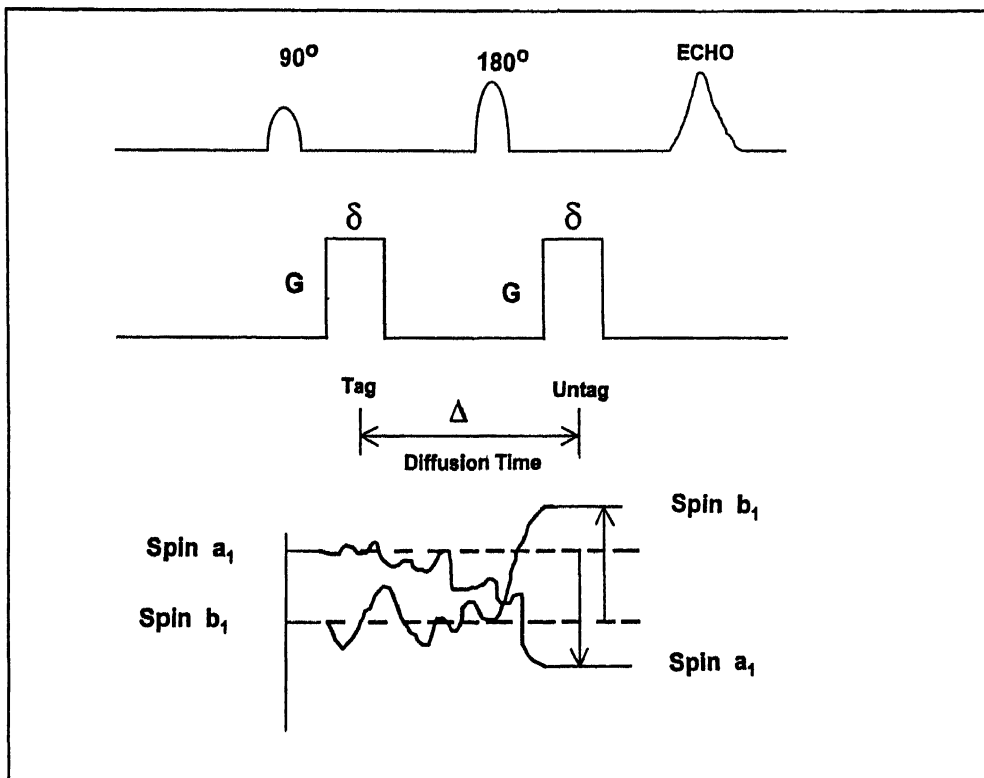


Fig 8—SE sequence with bipolar gradient pulses (See next for details)

diffusing spins which have different displacement histories (i.e., different phase shifts). Interference between their phases results in an attenuation S/S_0 (< 1) of the signal amplitude which, for the diffusion process, is given as⁵⁸

$$(S/S_0) = \exp [-(\gamma G \delta)^2 < L^2(\Delta) > / 2] \\ = \exp [-(\gamma G \delta)^2 \Delta D], \text{ from eq. (9).} \quad \dots (11)$$

Diffusion coefficients, or diffusion lengths, may be evaluated by varying either G or δ and by measuring the slope of the semi-logarithmic plot of (S/S_0) vs. $(G\delta)^2$.

There are two limitations to this simple diffusion model. Firstly, δ may not be negligible compared with Δ , in which case diffusion during application of the gradient pulses will be non-negligible. This is particularly true when using commercial clinical MR units which generally have only modest gradient amplitudes, thus necessitating long δ 's to produce appreciable diffusion effects. Secondly, unwanted background gradients may be present from the imaging sequence used. A more general formulation has therefore been used in the MRI context⁵⁹⁻⁶¹ whereby the signal attenuation becomes

$$(S/S_0) = \exp (-b D) \quad \dots (12)$$

where b is the 'gradient factor' characterising the strength and duration of the applied diffusion gradients and is defined as

$$b = \int_0^{TE} |k(t)|^2 dt \quad \dots (13)$$

with

$$k(t) = \gamma \int_0^t G(t') dt' \quad \dots (14)$$

where $G(t)$ represents the time evolution of all the gradients applied during the time that the spin magnetisation lies in the transverse plane. We note that the sign of $G(t)$ is reversed for $t > TE/2$ in a SE sequence.

The concept of 'diffusion weighted imaging' has been in use for some time^{59,62,63} and several methods are available to implement it. The basic idea is to sensitise the MR signal to diffusion by incorporating strong gradient pulses within an imaging sequence. The generated images then become diffusion-weighted. For quantification purposes, it is necessary to assess the degree of diffusion-weighting, which is accomplished by

computing the b factor from eq. (12). The b factor should always be provided with the data to make results comparable, just as one quotes TE and TR values to indicate the degree of T_2 - or T_1 -weighting.

Various imaging schemes have been implemented for diffusion imaging. The spin echo 2D FT method is by far the simplest to run. Other schemes are the stimulated echo sequence⁶², variants of the steady state free precession technique^{64,65} and the EPI-based sequences^{66,67}. In tissues, diffusion is usually limited by water interactions and collisions with macromolecules and membranes, for this reason biomedical studies quote their values as Apparent Diffusion Coefficients (ADC's).

We have so far argued that increased water diffusion can be made to lead to increased attenuation of the MRI signal. Conversely, if diffusion becomes retarded or restricted, signal intensity on diffusion-weighted images will increase. This latter observation, made only minutes after experimental induction of stroke^{68,69} by arterial occlusion, has been rationalised in terms of the early stages of ischaemic injury etiology, in which a phase of cellular swelling, or cytotoxic edema, occurs. During this phase, fluid electrolyte homeostasis is disrupted and there is an influx of water from the more rapid diffusive milieu of the extracellular space into the more encumbered environment of the intracellular compartment, changing the relative proportion of water molecules in each environment. This effect is combined with a constriction of extracellular space and increased diffusive path tortuosity, associated with swelling cells⁷⁰. This acute-stage marker for ischaemic injury has been reconfirmed in clinical practice using DWI sequences for early diagnosis of stroke⁷¹. DWI is today recognised as one of the most sensitive indicators of early ischaemic damage in the brain.

In oblong biomolecular assemblies such as tubular and fibrous structures, diffusion is not isotropic. Since diffusion is rigorously described as a tensor, the diagonal terms D_{xx} , D_{yy} and D_{zz} will then represent molecular mobility along the main tensor axes x , y and z . The signal attenuation is then given by a modification of eq. (12), namely,

$$(S/S_0) = \exp \left[- \sum_{i=x,y,z} b_i D_{ii} \right]. \quad \dots (15)$$

Anisotropic diffusion has been demonstrated in the myelin-sheath fibres of brain white-matter⁷² and has been studied in developmental investigations⁷³. Moreover, breakdown of fibre anisotropy holds promise as a marker for important demyelinating diseases such as multiple sclerosis. Present research trends indicate that the diffusion contrast mechanism will play an important role in characterising several other organ diseases such as renal ischaemia^{65,74} and tumour viability⁷⁵.

(ii) *Perfusion MRI*

While molecular diffusion is the result of random thermal motion, perfusion is a microcirculation process which measures nutrient delivery to tissues, and is a critical aspect of vertebrate physiology. Perfusion has been imaged and measured in clinical practice for many years, primarily in nuclear medicine, and its potential is therefore already well known.

Recent approaches to perfusion MRI exploit the motion-sensitivity of the images using flowing blood as an endogenous marker. Assuming that water exchanges between blood and tissue are slow compared with the measurement timescale of ~10 ms, which seems a reasonable hypothesis for brain perfusion⁷⁶, these methods share some features with those using pure intravascular tracers. However, MR techniques also offer original insights into the problem of perfusion not addressed by conventional methods⁷⁷. Some of these have already been used clinically, although full validation awaits more detailed studies

7 Contrast Enhancement With Exogenous Agents

Although the MR properties of the tissues and their micro environment are themselves capable of providing tissue contrast, pharmaceutical manipulation of the signal intensities is necessary in a variety of clinical situations to improve contrast between tissues with similar relaxation times but disparate histologies in the normal and diseased states.

The basic principles of exogenous contrast agents have been reviewed⁷⁸. The mechanism of contrast enhancement is related to several parameters: the class of contrast media (paramagnetic or superparamagnetic), their concentration within the tissue, their compartmentalisation in various physiological

and pathophysiological spaces, and the weighting of the imaging pulse sequence used. Thus, formulations that shorten either the tissue T_1 or T_2 relaxation time have been devised. The T_1 shortening agents are hydrophilic chelated paramagnetic metal ions such as Gd^{3+} and Mn^{2+} that have unpaired electrons and behave as molecular magnetic dipole moments. The fluctuating magnetic fields caused by the tumbling molecular dipole moments alter the T_1 of tissue water protons. The paramagnetic ions may be chelated to a wide variety of molecules to reduce toxicity and to retard the free tumbling rates of the metal ion centres thereby enhancing their relaxation effects on the tissue protons being imaged.

The second class of contrast agents alters the tissue T_2 (or T_2^*) values by inducing local magnetic field gradients in cells. As the tissue water protons diffuse through these induced field gradients, their MR signal is attenuated. Superparamagnetic materials such as magnetite particulates are representative of this class⁷⁹; as per one well-understood mechanism, they are taken up by phagocytosis and clustered within lysosomes intracellularly. Their value in vascular contrast enhancement has also been assessed recently⁸⁰. Several superparamagnetic iron oxide formulations (SPIO, USPIO) have been used as T_2 contrast agents for enhancing liver, spleen and bone marrow lesions because they are selectively absorbed by the reticulo-endothelial systems of the tissues.

In terms of their biodistribution, all MR contrast agents may be broadly grouped as being intravascular, extracellular, or organ specific⁸¹. This grouping is based on the type of chelation, packaging (i.e., liposomal, viral, encapsulated) or coating (e.g., 'antigen-specific'⁷⁹) surrounding the paramagnetic ions or particles.

Gd^{3+} -chelates, administered intravenously either as a continuous infusion or as a bolus injection, are available both in ionic and non-ionic formulations. The most common ionic agent uses Gd^{3+} ion and diethylaminetriaminopentaacetic acid (DTPA) as the chelating agent. This is widely used in brain pathologies that alter the blood-brain barrier (bbb), and clinical utility is high in both neoplastic and non-neoplastic diseases⁸². In pathologies affecting the bbb, e.g., an infarct, leakage of the paramagnetic agent across the disrupted tight junctions of the capillary endothelial cells

delineates areas of increased cerebrovascular permeability. Disruption of the bbb caused by tumour, inflammation or edema is observed as an area of hyperintense signal on T_1 -weighted images.

The so-called *non-ionic* formulation of Gd^{3+} chelate virtually accomplishes the same contrast enhancement as described above. The non-ionic paramagnetic complex has no net charge to be counterbalanced at the expense of an increase of viscosity and osmolality, and this feature leads to the potential clinical benefit of higher dose injections.

Among a host of other documented clinical applications, we cite three representative examples of diagnoses involving the spine, the heart and the liver. We have recently reported the diagnosis of a vertebral body affected by tuberculosis (Pott's spine) in a 35-year old male patient⁸³. A paravertebral abscess in his spinal cord MRI showed a conspicuous rim enhancement after intravenous Gd^{3+} -DTPA administration, enabling the diagnosis of Pott's spine in this patient.

Regions of myocardial ischaemia and infarction may be evaluated similarly using intravascular contrast agents. A Mn^{2+} -containing contrast enhancement agent that becomes preferentially distributed in the myocardium has been shown to differentiate between occlusive and reperfused infarcts in an experimental model⁸⁴.

MR signal intensity changes in the liver following selective uptake of iron oxide particles by Kupffer cells or hepatocytes are being increasingly used for detecting hepatic masses⁸⁵. Other pharmaceuticals that have been developed and successfully screened for MRI of the liver are classifiable into those that target the extracellular space, the hepatobiliary system, and the reticulo-endothelial system⁸⁶. Monoclonal antibodies have also been envisaged for targeting iron superoxide particulate complexes to tumor cells, and much research is continuing in this area.

8 Endogenous Deoxyhemoglobin Contrast and fMRI

A whole new dimension of MRI opens up when it is realised that blood itself can serve as an ever-present, endogenous contrast by virtue of the fact that deoxyhemoglobin is paramagnetic and this changes to diamagnetic oxyhemoglobin as the blood becomes oxygenated. The paramagnetism of deoxyhemoglobin has a significant magnetic

susceptibility effect leading to a local shortening of T_2^* . In 1982, Thulborn *et al.*⁸⁷ discussed the effect of this on the relaxation of water protons in whole blood. Ogawa and Lee⁸⁸ invoked this blood oxygen level dependent (BOLD) contrast mechanism to account for the dark appearance of veins at high magnetic field strengths.

In subsequent years, the operation of this BOLD mechanism in blood vessel hemodynamics has been widely exploited in *in situ* MR imaging of the functioning brain (fMRI) when a routine mechanical (motor) task or even a higher order perceptual or cognitive task is taking place. Such extremely novel experiments are in turn concerned with a fundamental goal of neuroscience, namely, to understand the cortical division of labour. What are the primary functions subserved by particular groups of neurons of the brain, and how are these different groups recruited, coordinated and reorganised in the performance of complex brain operations?

Answers to the above questions are often sought from a fast gradient echo based MRI sequence which is sensitive to T_2^* (i.e., susceptibility related spin dephasing) effects. On T_2^* -weighted images, areas of magnetic field distortion are hypointense (dark)⁸⁸. Deoxygenated blood, being paramagnetic, leads to such a signal attenuation in the 'resting' capillary bed (Fig. 9a). During a task-related neuronal activation, some neurons require additional energy and therefore induce a dilation of the brain capillaries. There is a focal increase in the cortical blood flow and, to a lesser extent, the oxygen extraction, in the brain areas mediating the task performance. Due to the increased regional blood flow a larger quantity of non-saturated ^1H spins enters the activated area, enhancing the signal intensity ('inflow' contrast phenomenon). On the other hand, during activation the increase in the regional blood flow overcompensates for the oxygen extraction, and therefore the activated brain area experiences a relative decrease in deoxyhemoglobin, leading to less susceptibility distortions (Fig.9b). As a result, T_2^* becomes longer in the activated area and signal intensity is enhanced. Dynamic tracking of this phenomenon with high-speed, T_2^* -sensitive imaging is the basis of fMRI.

The respective signatures of 'inflow' and 'BOLD' contrast may often overlap, leading to an overall signal enhancement of 1-5% at 1.5 T. At a

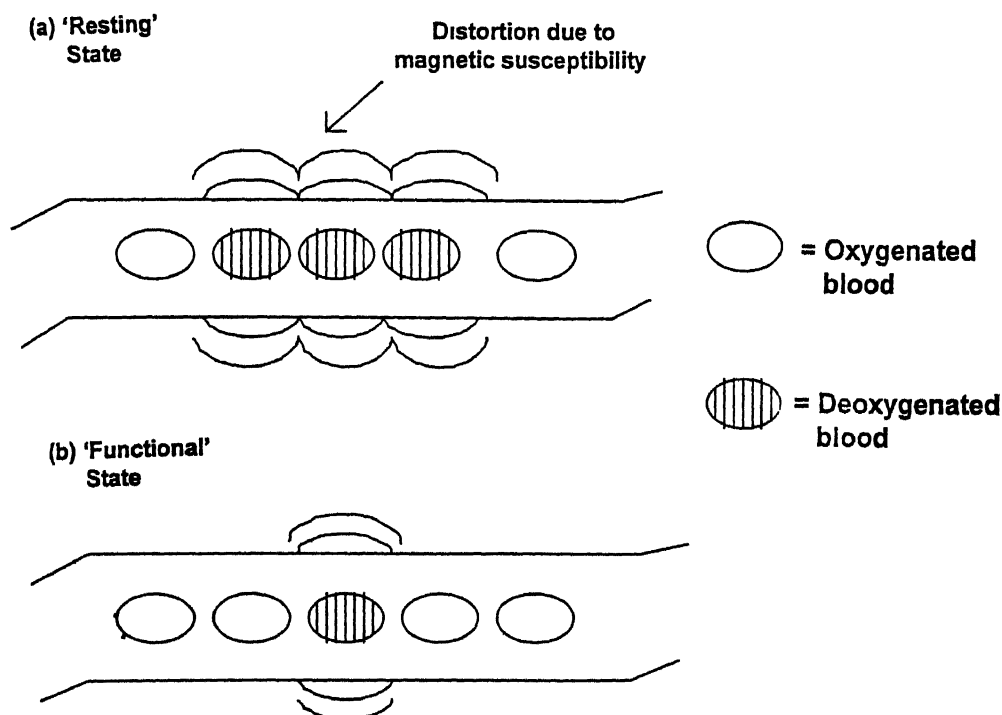


Fig 9 Blood Oxygen Level Dependent (BOLD) contrast . (a) paramagnetic deoxygenated blood leads to signal attenuation in the 'resting' capillary, (b) increased regional blood flow in the 'functioning' capillary gives signal enhancement

higher (say, 4 T) field, the susceptibility effects (being field-dependent) predominate, leading to a significantly higher (5-20%) signal. However, the implementation of 4 T imaging is more complex in the clinical setting, and most of the clinical applications will probably be achieved at 1.5 T or 2 T.

Functional MRI is essentially a subtraction technique involving difference maps between the 'Activated' and 'Rest' states. Initial experiments have been successful in studying activation of the visual⁸⁹⁻⁹¹ and motor/sensory⁹² systems. Task-activated motor cortex stimulation in resting volunteers has also been mapped by fMRI in our laboratory⁹³ and elsewhere^{94,95}, and these studies together demonstrate the involvement of the primary (M1), supplementary (SMA) and pre-motor (PM) areas during complex and imagined muscle activation.

According to neurophysiological concepts, higher cognitive brain functions such as language and intelligence are no longer attributable to single centres of relative functional autonomy and specialisation, but are rather represented by dynamic networks of cortical and subcortical structures⁹⁶. These are really green pastures for

research, and the results could often be startlingly novel. In the case of a volunteer who was asked to solve a pegboard puzzle, fMRI showed the bilateral involvement of the dentate nucleus of the cerebellum⁹⁷, suggesting that in addition to its well-established role in motor movement, the cerebellum is also active in cognitive functions. Gender differences in the functional organisation of the brain for language were demonstrated by fMRI, and illustrated a higher lateralisation in males⁹⁸.

Techniques of fMRI have also aided certain brain pathologies. In patients with brain tumours, identification of functional areas of the cortex by fMRI prior to surgery compared very well with the 'Wada test' which consisted of the intracarotid injection of amobarbital⁹⁹. Similarly, fMRI maps confirmed the intraoperative electrocortical stimulation maps in patients worked up for epilepsy requesting temporal lobectomy¹⁰⁰.

It is clear that the future of neuroimaging will include both anatomical MRI and fMRI. The ability to identify a region of suspected pathology in individual brains with great precision and to superimpose detailed function (on a scale of *millimeters* of resolution and *milliseconds* of temporal activity) tests the limits of current

technology. Computer hardware has been evolving in the direction of more standardised platforms (e.g., RISC processors running UNIX) as imaging instrument manufacturers tap into the increasing accessibility of inexpensive and powerful workstation systems. Also, as massive parallel supercomputers become increasingly available, their use in fMRI will lead not only to a finer level of diagnostic image quality but also to new insights into the real-time activities of the stimulated brain.

9 Heteronuclear MRI and Hyperpolarised Spins

Some MR imaging experiments using nuclei other than ^1H (primarily ^{19}F , ^2H and ^{23}Na) directed towards the measurement of functional tissue parameters have also been described. Such measurements are exacerbated by the low intrinsic sensitivity of these nuclei relative to ^1H and the low physiologic concentrations of signal-producing material relative to water. ^{23}Na MRI studies have demonstrated that the method can be useful for monitoring edema development and the cerebral redistribution of Na following ischaemia^{101,102}.

^{19}F MRI methodology depends on the exogenous addition of a fluorinated material, since no ^{19}F exists *in vivo*. Perfluorocarbons that label vascular compartments at high concentrations have been most commonly used, especially in the assessment of tumour vascularity¹⁰³ and in the kinetics of tissue perfusion¹⁰⁴. Furthermore, the ^{19}F relaxation times of these compounds are sensitive to oxygen concentration, and this has been the basis for a novel method of using a fluorocarbon blood substitute and measuring the local oxygen tension in living tissue noninvasively¹⁰⁵. ^{19}F MRI of fluorinated glucose and its metabolites has also been exploited in an experimental study of regional carbohydrate metabolism¹⁰⁶.

The measurement of regional cerebral and tumoural blood flow by serial ^2H MRI after bolus arterial injections of deuterated saline also appears to be a promising methodology^{107,108}. Methods of utilising ^2H MRI to follow regional differences in metabolic processes involving rather highly concentrated metabolites in the kidney have also been explored¹⁰⁹.

At body temperature, only a small fraction (10 per million) of spins are polarised, and the induced voltage in the MRI receiver coil is minute. Recently, techniques to circumvent this problem

have been developed using coherent laser radiation to hyperpolarise the nuclear spins in ^3He and ^{129}Xe ¹¹⁰⁻¹¹². By this means a gain of 10^5 in spin magnetisation can be achieved, which promises to lead rapidly to the visualisation of pulmonary ventilation by MRI, particularly since the human lungs have notorious limitations with regard to observing imageable protons.

10 *In vivo* MR Spectroscopy (MRS) and Tissue Metabolism

In 1974 it was demonstrated that phosphorus containing metabolites could be detected in intact muscle tissue with the use of ^{31}P MR spectroscopy¹¹³. This work may be considered the harbinger of all the present-day MR spectroscopic approaches to *in vivo* human biochemistry.

(i) Importance of Spectral Localisation

For *in vivo* MRS to be useful, it is mandatory that the spectral data originate only from an appropriately chosen volume of interest (VOI). Isolating the VOI from the rest of the tissue for acquiring MR spectra is called *localisation*. Techniques of localisation and selective RF excitation of a localised voxel *in vivo* constitute a vast area of methodological development, and excellent reviews are available^{114,115}. Rather than enumerating all the methodologies available, we shall only survey the fundamental principles of those that are more commonly used.

(ii) Surface Coils

Historically, the first device employed to overcome the poor signal-to-noise ratio of the low-concentration tissue metabolite signals was the surface coil RF antenna. Introduced in 1980¹¹⁶, the surface coil's utility in MR spectroscopy has since been discussed thoroughly¹¹⁷. Consisting of just a one- or two- turn copper loop, it is the simplest type of MR probe for localised signal reception, which is easily positioned on or under a patient's organ (chest, spine, abdomen, orbit, breast). It is not only the geometry of choice for ROI's nearer the body surface, but also has the property of providing a degree of spatial isolation.

Surface coils can be either 'transmit and receive' or 'receive only' RF antennas. Frequently, another coil or volume resonator with a more uniform RF profile is used to transmit, and the surface coil is used for reception. The non-uniform distribution of

the B_1 excitation energy emitted by a surface coil transmitter¹¹⁷ typically means that an RF pulse will result in a distribution of flip angles over the interrogated volume. Non-uniform B_1 fields arising due to such a distribution can be reduced through the use of adiabatic RF pulses^{118,119}

Results from surface coil studies must be interpreted with caution; when the coil is positioned to receive signals from nonhomogeneous regions of tissue, there is always the possibility of signal contamination arising from unwanted ROI's. An important early strategy to overcome this inherent problem with surface coil receivers was the depth resolved surface coil spectroscopy (DRESS) which successfully localised ^{31}P and ^1H *in vivo* signals¹²⁰. In the DRESS sequence, a shape-selective RF pulse applied to the surface coil in the presence of a G_y gradient (directed perpendicular to the coil surface to provide depth selection) excited MR signals from desired regions of interest.

(iii) Image-Guided Protocols and Water Suppression

Most current MR localisation methods are

'image guided' in that they use ^1H MR images to guide the placement of regions of VOI for RF excitation. Therefore, spectroscopy protocols typically include an MRI study¹²¹. The building-blocks of an image-guided and volume-localized MRS protocol are shown in Fig. 10. Steps 1 to 5 in this figure describe the scout imaging procedure required for guidance, step 6 involves voxel selection, and step 7 refers to the important water suppression procedure (described below). Steps 8 to 10 depict the rest of the procedures involved in recording an MRS spectrum.

Before surveying the various signal localisation techniques, one ought to discuss water suppression. Detection of resonances from biochemicals with low concentrations in the presence of a large water proton signal imposes severe dynamic range constraints on the analogue-to-digital convertor of the MRS system. Since the concentration of water in tissue ($\sim 70\text{ M}$) is orders of magnitude greater than the tissue concentration of most metabolites of interest ($\sim 10\text{ mM}$), the water signal will first have to be suppressed a thousandfold before the metabolite signals are recorded from the chosen voxel. Several water suppression techniques take

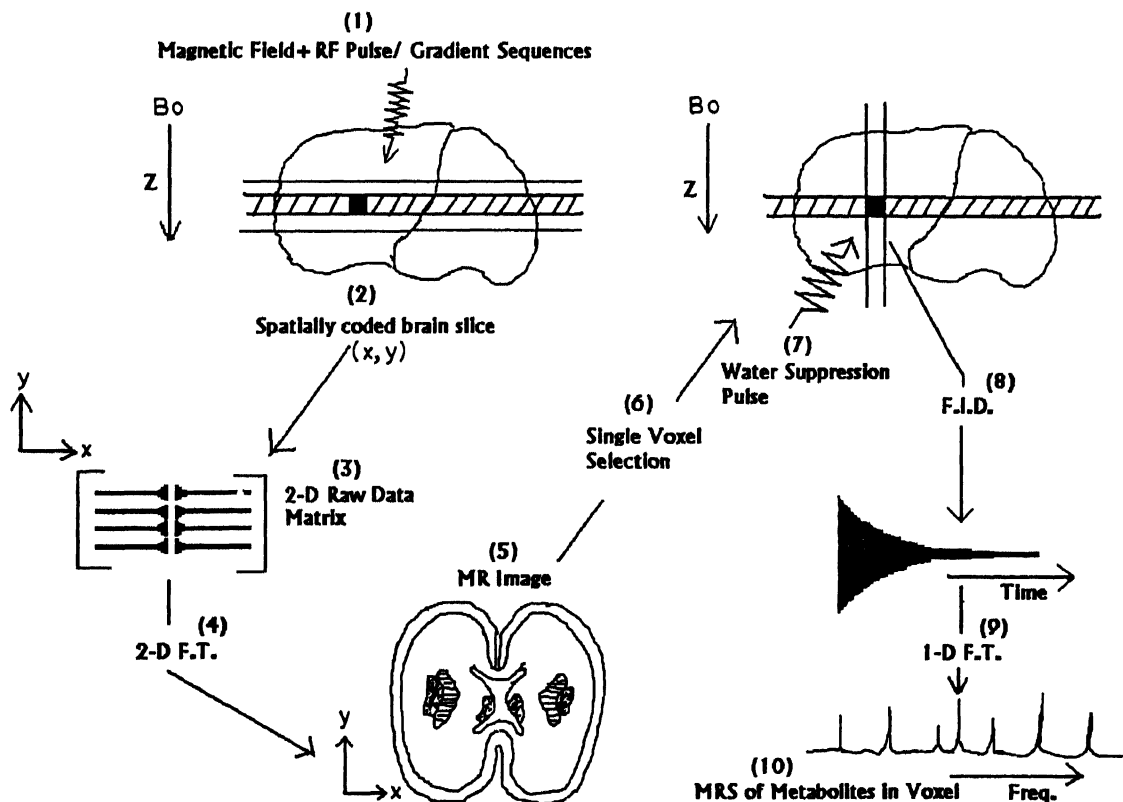


Fig. 10 Building blocks of an image-guided, volume-localised and water-suppressed *in vivo* MRS protocol (Adapted from Ref 121)

advantage of the chemical shift differences between water and other metabolites¹²²⁻¹²⁴. The most common approach is to use chemical shift selective (CHESS) RF pulses¹²⁵ constructed to excite a limited narrow band (~60 Hz) of RF frequencies corresponding to water in the sample. One or more 90° RF excitations of water are followed by dephasing 'crusher' gradients to eliminate the excited water signal in the transverse plane. While the water magnetisation is beginning to recover and is not available for excitation, the rest of the metabolites in the selected ROI are excited and measured.

(iv) *Single Voxel Localisation*

One very successful strategy for spectral localisation is to apply a series of slice-selective RF pulses so that only the spins at a localised VOI are resonated to produce a signal. The position and size of the VOI are determined by the region bounded by the intersection of the three orthogonal slice planes. The shape thus defined is considered as being a box, but the actual shape would normally depend on the slice profiles of the selective pulses. Three principal variants of the volume localisation scheme are discussed below

(a) *PRESS*

The Point-Resolved Spectroscopy (PRESS) scheme¹¹⁴ involves a double spin-echo sequence in which three mutually perpendicular slices are excited by one 90° and two 180° sinc-shaped RF pulses. Only the spins in the volume defined by the intersection of the three excited slices experience all three pulses and create a second echo at a time $t = 4\tau$ (where τ is the time between the 90° pulse and the first 180° pulse) and only this second echo is refocussed and acquired. Each 180° pulse generates a SE signal from a whole line of the sample that intersects the excited plane, whereas signal from only the small selected volume at the intersection of all the three planes is desired. For this reason, and also for the reason that imperfections in the 180° pulses may produce spurious transverse magnetisation, it is advantageous to add large gradient 'crusher' pulses to eliminate signals from outside the selected volume

The initial 90° pulse produces a transverse magnetisation, and during the entire sequence this magnetisation remains in the xy plane and

contributes to the measured second-echo signal. The slice profiles of the 180° pulses are often worse than those of a 90° pulse, and consequently PRESS VOI definition and spectral localisation may be worse than those of the other two schemes to be described below. Further, the repeated 180° pulses require more power than 90° pulses, which may be in excess of clinical safety limits for either large VOI's (i.e., thick slices) or large objects.

For ³¹P and ¹³C *in vivo* MRS, the additional SE pulses and delays in PRESS have the drawback of increasing the time between the initial RF excitation and the final acquisition to a point where a serious loss of metabolite signals due to T_2 processes may become inevitable. However, for ¹H spectroscopy, where T_2 's are much longer, useful applications to brain MRS have been demonstrated when combined with water suppression methods.

(b) *STEAM*

The Stimulated Echo Acquisition Mode (STEAM) spectroscopy¹²⁶ involves the successive excitation of three slices by applying three selective RF pulses each in the presence of an orthogonal gradient. The localisation sequence, combined with the water suppression pulses (WS1-WS3), is shown in Fig. 11. The localisation consists of three slice-selective RF pulses (P1-P3) along with selective (SS), refocussing (R) and crusher (CR) gradients. Pulse P1 in the presence of the G_z gradient excites a slice normal to the z -axis. Pulses P2 and P3 in the presence of G_y and G_x , respectively, excite two other orthogonal slices. At time $TE/2$ (TE being the echo time) following the third RF pulse, the transverse magnetisation from the ROI becomes refocussed giving rise to the stimulated echo. The position of the ROI can be varied by changing the central frequency of the RF pulses, while the dimensions of the ROI 'box' can be varied independently in any direction by adjusting RF pulse bandwidths and/or the strength of the gradients.

While the STEAM sequence is conceptually simple to understand, certain further features related to its successful implementation ought to be reviewed. The three RF pulses used in this sequence generate not only the desired stimulated echo originating from the ROI but also a number of unwanted coherences. The rigorous theory of the coherence pathways underlying the 'stimulated echo' formation¹²⁷ need not concern us here but,

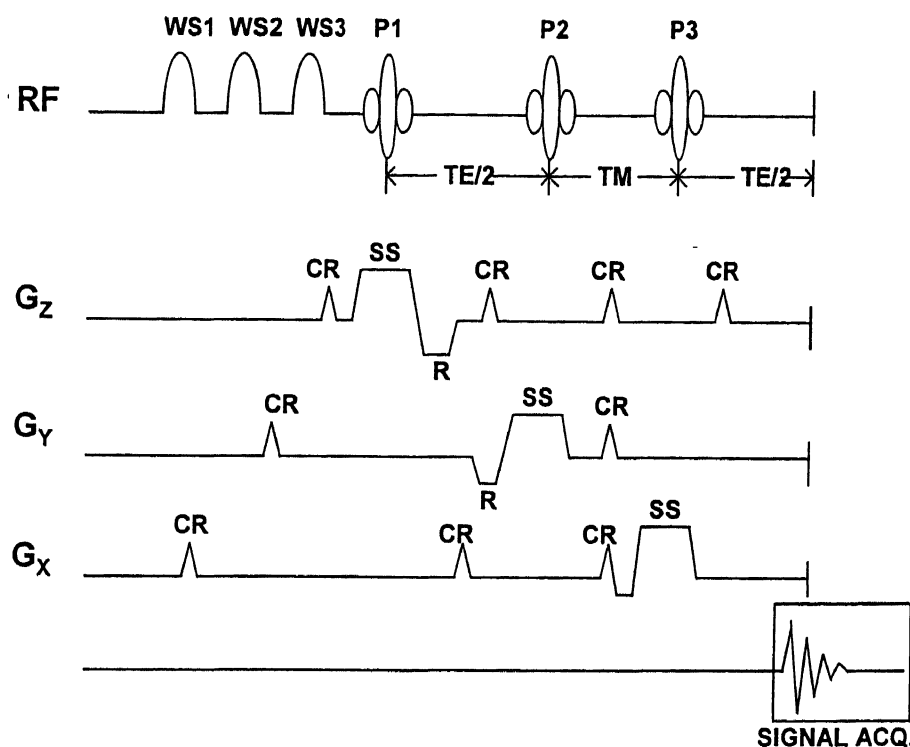


Fig 11 The STEAM pulse sequence for water-suppressed, volume-localised MRS. Pulses P1 to P3 localise the ROI while water suppression is done by pulses WS1 to WS3. Slice selective (SS), refocussing (R) and crusher (CR) gradients are discussed in the text.

for proper localisation, it is important to eliminate the contributions of all the unwanted coherences. This is accomplished by dephasing these coherences through the crushers applied during the $TE/2$ and TM periods (Fig. 11). This dephasing allows the second RF pulse to partition the resonating spins into a 50% transverse component and a 50% longitudinal component. While precessing during TM , the transverse component refocuses the phase information acquired during the first $TE/2$ interval and leads to a primary SE at $TE/2$ after the second RF pulse. During the TM period, only the phase-prepared longitudinal magnetisation will decay according to T_1 , but *not* T_2 -relaxation; TM is therefore kept short to avoid significant signal loss. The third RF pulse, P3, rotates this magnetisation back into the xy plane, where it refocusses after another $TE/2$ interval to give the STEAM signal which is accumulated in the analogue-to-digital converter (ADC).

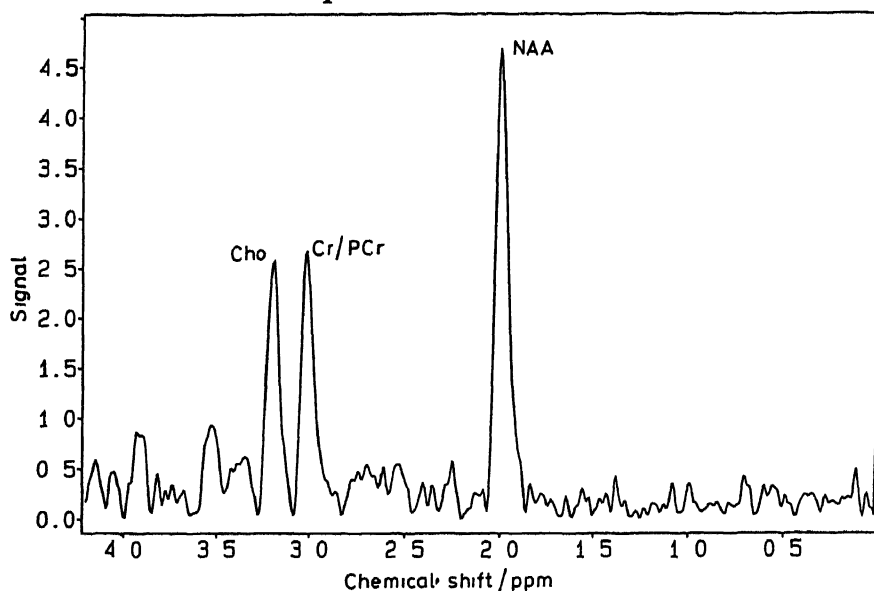
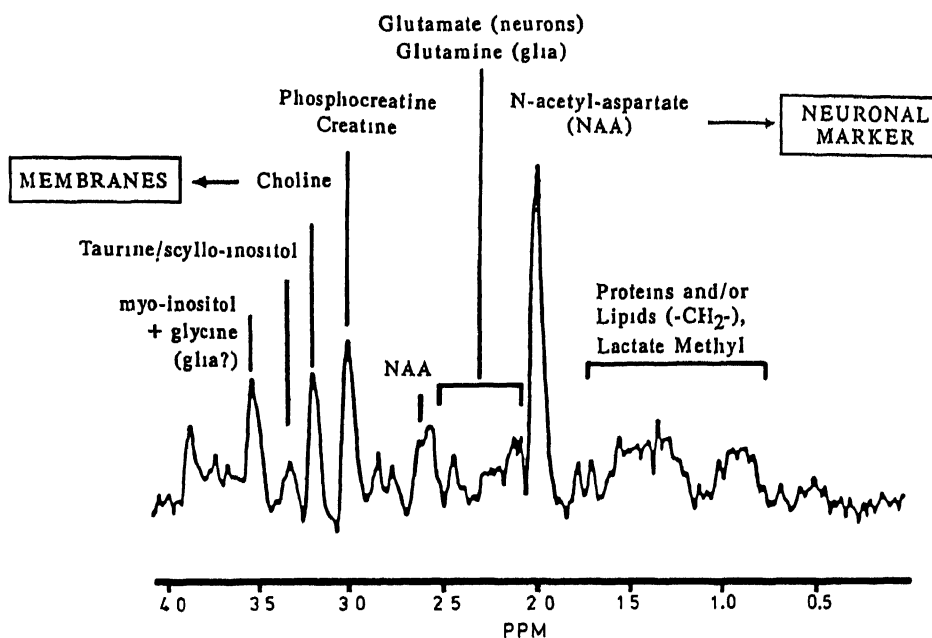
We thus see that only half the magnetisation for the ROI is manifested as the STEAM signal. This disadvantage notwithstanding, this sequence has excellent localisation properties¹²⁸. Also, the sequence enjoys other advantages. Flip angles (α) of pulses P1-P3 are not crucial for localisation so

long as each of them is less than or equal to 90° . This reduces the run time of the protocol. An important consideration in the clinical setup is the exposure of tissues to RF power, which can also be kept low by using $\alpha < 90^\circ$ RF pulses since the deposited RF power varies as α^2 .

For the above reasons, the STEAM sequence is very widely used today for *in vivo* ^1H MRS studies. An important pulse sequence parameter influencing the information content in such studies is TE . Long TE (typically 136 or 272 ms) ^1H spectra contain signals from the cholines ('trimethylamino' head group), Cho, at 3.2 ppm, creatine/phosphocreatine (Cr/PCr) at 3.0 ppm and N-acetylaspartate (NAA) at 2.0 ppm.

A sample 'long- TE ' (135 ms) ^1H spectrum from our work on a normal volunteer is shown in Fig. 12. Under certain pathological conditions (several examples of which will be reviewed in a later section), elevated lactate and mobile lipid backbone signals may also be detected at 1.33 ppm and 1.30 ppm, respectively. Long TE 's may complicate the straightforward interpretation of signals from compounds having strongly spin-coupled groups, such as the $-\text{CH}_2-$ groups of glutamate and glutamine. The increased TE 's may

Proton spectrum from normal brain

Fig. 12 Representative 'long-TE' STEAM ^1H MRS spectrum from the brain of a volunteerFig. 13 Representative 'short-TE' ^1H MRS of STEAM localised brain white matter

lead to spin-dephasing resulting from J -modulation of the multiplet resonances. At short TE 's, however, the metabolite peaks are seen as J -coupled multiplet patterns because these multiplet components have less time to dephase in accordance with J -modulation. Also, by employing much shorter TE 's (30 ms or less), a number of signals from short- T_2 metabolites may appear in the spectrum, e.g., myo-Inositol and glutamine/glutamate. In Fig. 13 is shown an

illustrative ^1H MRS of a prototypical $TE=20$ ms, single-voxel localised brain white-matter. The features identified in this figure shall be further used in our discussions later on.

(c) ISIS

The Image-Selected *in vivo* Spectroscopy (ISIS) scheme¹²⁹ also enables a spectrum to be acquired from a single voxel. The experiment involves a preparation period during which the longitudinal

magnetisation is excited by means of selective inversion pulses applied in presence of gradient pulses. The preparation period is followed by a delay, allowing time for eddy currents (in the conducting magnet structures) to decay. Finally, the longitudinal magnetisation is sampled by applying an 'observe' pulse which can be a pulse sequence designed to perform T_1 or T_2 measurements, water suppression, spectral editing, etc. To achieve a VOI, a set of eight acquisitions is performed, each consisting of a different combination of inversion pulses, i.e., a 'phase cycle'. The RF receiver is inverted when an odd number of selective pulses is applied. Addition of the eight acquisitions results in coherent averaging out of signals from the regions outside the VOI. Since the method is based on an add/subtract process, one ought to ensure that the magnetisation is the same at the beginning of each experiment. This is done by appropriately optimising the phase cycling.

ISIS is not T_2 -weighted. Unlike STEAM and PRESS, which combine localisation and acquisition pulses, ISIS achieves localisation before the excitation sequence, allowing total echo evolution times (TE+TM) to be minimised. This reduced time maximises signal-to-noise ratio by minimising T_2 and J -modulation losses. This is a major advantage for *in vivo* ^{31}P MRS where short T_2 's are encountered.

(v) Multi-Voxel Localisation: Spectroscopic Imaging (SI)

Spectroscopic Imaging (SI) or Chemical Shift Imaging (CSI) are the terminologies used to describe multi-voxel MRS acquisitions that employ phase encoding of spatial position^{130,131}. Here the spatial resolution is introduced via B_0 phase encoding, i.e., using B_0 gradients which encode positional information into the *phase* of the MR signals. Since the signals are themselves detected during the absence of B_0 gradients, the chemical shifts modulate the signals along the spectroscopic axis. Similar to phase-encoding in MR imaging, a sequence of gradient values generates a complete set of spectra which can be spatially and spectroscopically resolved after multi-dimensional FT. The result is an (n+1)-dimensional (namely, n-D spatial and 1-D spectroscopic) map.

A distinct advantage of SI over single-voxel techniques is its ability to give a better definition of

the regions where the spectra are being measured. Additionally, the localized spectra from many locations are acquired simultaneously, giving a larger detected signal and better signal-to-noise ratio. Instead of only averaging N different signals from a single voxel, the signals from an entire region are obtained N times, each with a different phase encoding.

By way of illustration, we show the ^1H multi-voxel SI acquired from a patient with intracranial tumour (Fig. 14). At the top left of this figure is the frequently displayed array of voxels, each with the phase encoded spectra of the tumour region, and the 1-D metabolite spectrum of a single phase-encoded scan is shown at the top right. An alternative display mode is to restrict the information content to one (grey scale) or a few (color) measured chemical species and to construct an image (distribution map) from the peak area under its signal; one such grey scale image (NAA) is shown at the bottom left-hand side of Fig 14, while the bottom right corner demonstrates the use of a contour map to represent a metabolite level.

A major limitation of SI stems from the fact that the limited number (say, 16 or 32) of phase-encode steps means a limited sampling density. In k -space, this results in 'Fourier bleeding', causing a decreased localisation and signal contamination from outside the VOI. Another limitation of SI is that constraints on spatial and spectral resolution and bandwidth, and imaging time, are set by the k -space trajectory used, rather than the intrinsic imaging parameters of voxel size, integration time and input signal-to-noise ratio. A flexible pulse sequence has been described¹³² which obviates most of the above constraints.

Recent technical improvements of SI include (i) 3-D echo planar SI performed at short TE's in the human brain¹³³, (ii) double-echo multislice ^1H SI using Hadamard slice encoding, i.e., encoding the spatial information on slices using the ± 1 elements of the Hadamard matrix¹³⁴, and (iii) the hybridisation of the two localised spectroscopy techniques, namely, SI and Hadamard Spectroscopic Imaging, to obtain proton-decoupled ^{31}P spectra from the human brain¹³⁵.

11 Representative Applications of *in vivo* MRS

In vivo MRS has been the subject of extremely vigorous clinical and biomedical investigations over the past decade. Although the technique

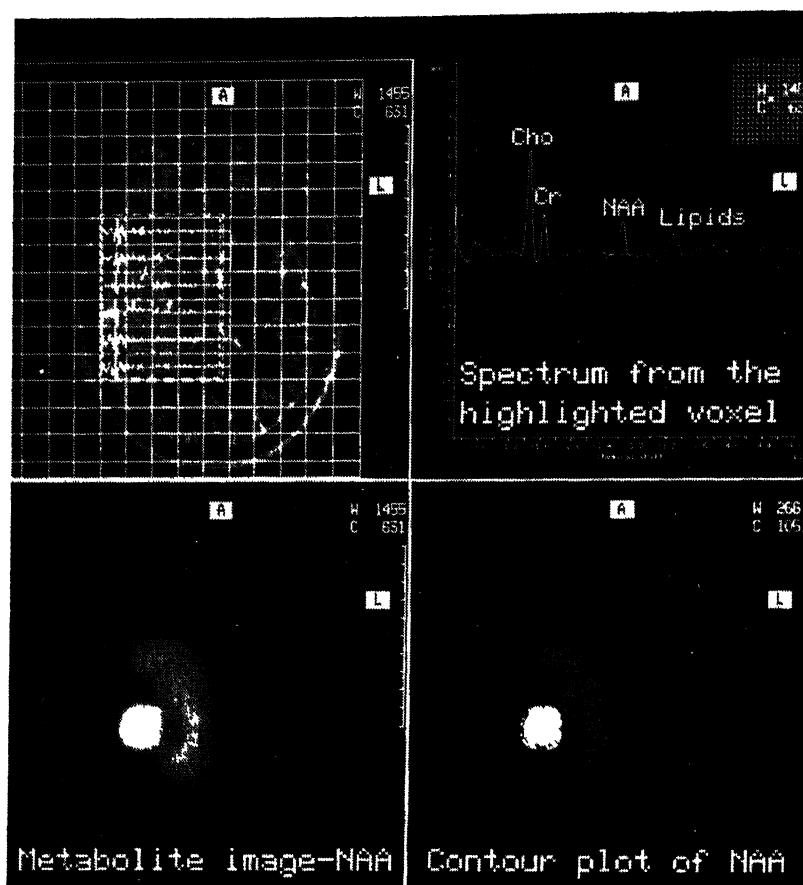


Fig 14 Proton Spectroscopic Imaging of brain (axial slice) of a patient with intracranial tumour Top left Voxels with phase encoded spectra of the tumour region Top right 1-D Metabolite spectrum from the highlighted voxel Bottom left Grey-scale metabolite image of NAA, Bottom right Contour plot of NAA

provides a wealth of information regarding biochemical processes underlying normal and diseased tissue, these measurements could often be technically demanding. Using the various robust procedures surveyed in the previous section, clinically 'feasible' MRS examination times during which a patient may remain motionless and comfortable inside the magnet tunnel can produce very relevant and important information. We shall survey the work published over the last decade which typifies the clinical trends in the areas of ^1H , ^{31}P and ^{13}C MRS.

(i) ^1H MRS of Brain Metabolites

Proton MR spectroscopy of the human brain is gradually emerging as a technique for differential diagnosis^{30,136,137} and useful clinical applications are being reported¹³⁸. Every major neuropathology¹³⁹ as well as certain forms of

addiction¹⁴⁰ and several less common conditions related to the brain¹⁴¹ and spine⁸¹ have been explored using proton MRS.

The focus of most of the work in ^1H MRS of the early nineties has been on the diagnosis and staging of brain tumours. At 1.5 T, the observable ^1H chemical shift range of interest is a somewhat crowded one, normally falling within a 6 ppm (or 380 Hz) range. Although not completely definitive, ^1H MRS may allow the distinction between low-grade and high-grade primary brain tumours gauged by the elevation of choline, reduction in NAA and creatine, or elevation of lactate¹⁴². Basic disease diagnostics rest on the following metabolite signals.

(a) Amino Acids and Intermediary Metabolism

The acetyl group of the amino-acid, *N*-acetyl aspartate (NAA), causes the major resonance in a water-suppressed ^1H MRS (Fig.12). NAA is

believed to be present only in neurons and axons, and has been observed to be reduced in many conditions which involve neuronal loss such as infarcts, brain tumours, seizure foci, multiple sclerosis, dementia (Alzheimer's disease), and other neurodegenerative processes. Because of its largely neuronal location, NAA is generally accepted to be a specific and putative *in vivo* marker in the adult-brain in stroke and epilepsy¹⁴³. Neuronal loss (heralded by NAA peak area reduction) in grey matter, particularly in the frontal lobe cortex of the brain, has been claimed to be a clinically eloquent diagnostic for HIV, while important single voxel ¹H MRS experiments have been demonstrated for the differential diagnosis of focal brain lesions in AIDS patients¹⁴⁴. Interestingly, NAA can also be found in some neuroglial precursors (such as 0-2A progenitors) and immature oligodendrocytes¹⁴⁵, suggesting its involvement in lipid synthesis during myelination.

The *choline* -N(CH₃)₃ signal at 3.2 ppm, usually denoted by Cho, consists of compounds (glycerophosphocholine, phosphocholine and phosphatidylcholine) which are involved in membrane synthesis and degradation. The Cho signal has been observed to be elevated in glial cells, brain tumours, and demyelinating diseases such as multiple sclerosis and leukodystrophies. Response to a therapeutic regimen may be followed by monitoring the decline in Cho levels, which is most effective if a pretreatment baseline examination is done¹⁴⁶.

Glutamate, a highly concentrated amino acid, and the glutamate-related free amino acids such as glutamine, aspartate and γ -aminobutyric acid (GABA), can be detected during the short-TE MRS experiment (Fig.13). Glutamate is a key metabolite involved in neurotransmission, Krebs's cycle, brain ammonia regulation and fatty acid synthesis. Other amino acids, sometimes detected by ¹H MRS, are directly involved in excitatory (aspartate, glycine) or inhibitory (GABA, taurine) neurotransmission, and sometimes in excitotoxicity (glutamate, aspartate). Changes in glutamine and myoInositol (mI) have also been reported in pathologies such as hepatic encephalopathy¹⁴¹ and Alzheimer's disease¹⁴⁷.

The *GABA* resonance in *in vivo* ¹H MRS usually overlaps with glutamine and glutamate resonances but it can be selectively observed by using spectral editing techniques¹⁴⁸. Reduced GABA activity is an

important manifestation in the pathogenesis of epilepsy, contrarily, GABA can be detected more easily under nonphysiological conditions when it is increased, e.g., during an antiepilepsy treatment¹³⁷.

The >N(CH₃) group of *creatine/phosphocreatine* (Cr/PCr) yields a strong signal at 3.03 ppm (Fig 12). Minor contributions to the intensity of this peak may come from GABA, lysine, and glutathione. The resolution achieved between the sharp Cr/PCr peak and the nearby Cho peak is usually an indicator of a well-shimmed, homogeneous magnetic field over the ROI. An additional creatine peak can be observed at 3.94 ppm.

Although creatine by itself is of little biochemical interest, the equilibrium enzyme creatine kinase (converting Cr to PCr) appears to be crucial to the maintenance of energy-dependent systems in all brain cells¹⁴⁹. Creatine may increase in hypometabolic tumours but decrease in hypermetabolic tumours¹⁴⁶.

(b) *Lactate Metabolism*

Since the lactate signal is not seen in the ¹H MRS of normal brain, its appearance is often an important marker of tissue status and deranged energy metabolism. Lactate has been observed in ischaemia, hypoxia, brain tumours, mitochondrial disease and other conditions.

In tumour studies lactate is more likely to be found in high-grade gliomas, but its presence is not a reliable indicator of malignancy¹⁴⁶. Lactate measurements will be more crucial, for example, in human stroke. The appearance of a sizeable signal at 1.33 ppm, arising from the lactate CH₃ group, is usually taken to be a necessary, but not sufficient, condition for establishing lactate elevation. This is because the lipid -CH₂- signal emanating from nearby regions (e.g., skull fat) can overlap this spectroscopic region. Fortunately, lactate is a coupled AX₃ Spin System, and the *J*-coupling (~7 Hz) between the CH₃ and methine (CH) protons of lactate can be exploited to modulate the well-known zero-^{150,151} and double-quantum¹⁵² coherence signals of lactate.

In vivo ¹H MRS has the potential to provide a noninvasive differential diagnosis between brain abscess and tumour^{153,154}. Often, the former pathology may be indicative of the immunosuppressive state of the patient. Brain abscess exhibits its own spectral signatures from a

number of markers of infectious involvement, such as acetate and succinate¹⁵³.

(c) Lipid Metabolism

The 1.3 ppm $-\text{CH}_2-$ backbone signal (and the corresponding 0.9 ppm $-\text{CH}_3$ end group signal) often arise from mobile lipids^{30,155,156}. In normal brain, $-\text{CH}_2-$ and $-\text{CH}_3$ signals from acyl chains of lipids cannot be detected. These resonances do manifest themselves, however, when mobile lipids accumulate in the brain under abnormal conditions such as paroxysmal disorders¹⁵⁷ or from the presence of macrophages, bacterial strains¹⁵³, inflammatory cells, edema, neoplastic cells or damaged membranes of degenerative cells (in the last mentioned case, blood-brain barrier damage will be involved which can be confirmed by Gd^{3+} contrast enhancement in the MR image)

Very recently, we have used localised ^1H MRS for demonstrating biochemical abnormalities related to myelin maturation in different brain regions of patients with congenital hypothyroidism (cretinism) before and after thyroxine treatment¹⁵⁸. Thyroxine therapy reverses the myelin-related abnormalities, which is an important result considering that the therapy was initiated at ages beyond which myelinogenesis abnormalities are usually considered irreversible

(iii) Functional ^1H MR Spectroscopy (fMRS)

MRS during the actual course of functionally activating the brain motor cortex of volunteers has been reported by us recently^{159,160}. From the combination of lactate and glucose changes observed during a T_2^* -weighted BOLD imaging in the activated human visual cortex, Frahm *et al.*¹⁶¹ have suggested a decoupling of the roles of oxidative metabolism and anaerobic glycolysis during different phases of prolonged photic stimulation.

A novel spectroscopic approach to the measurement of functional activation has been demonstrated by Hennig *et al.*¹⁶² who have performed a non-water suppressed localised MRS experiment using the PRESS sequence to study signal changes of the water resonance during cortical activation by visual, motor and median nerve stimulation.

(iv) ^{31}P MR Spectroscopy

^{31}P MRS is particularly suited to the study of

muscle energetics in a variety of *in vivo* situations (rest, exercise, fatigue, training, effects of diseases, therapy, etc.). Of prime importance is the supply and expenditure of ATP (the body's main energy currency) and how its utilisation pathway is controlled. Two often-studied indices are (i) the role of ADP as the primary regulator of oxidative phosphorylation and (ii) the rate of post-exercise PCr recovery as an index of muscle's oxidative capacity. Any pathological process that interferes with energy metabolism, be it ischaemia, toxic drugs or myopathies, may result in changes in intracellular PCr, ATP and inorganic phosphate (P_i), and these are precisely the compounds that have well-defined, chemically shifted MRS resonance peaks which are therefore easily detectable in the tissue noninvasively, without the need for biopsy.

Several metabolic peaks can be measured. PCr, at 0.0 ppm, is a repository for high energy phosphate groups used in metabolism and is often taken as the reference for chemical shifts because its resonance position does not change with local conditions. P_i provides a sharp peak at 4.8 ppm. Since the P_i resonance position is a logarithmic function of the free hydrogen-ion concentration, the shift of the P_i peak relative to PCr is often used⁷ as an indicator of tissue pH, and has been used for studying pH gradients across tumour cell membranes¹⁶³. Also, observations of lactate production in tumours by ^1H MRS often find a parallel in the corresponding ^{31}P experiment, where increase of lactic acid corresponds to a decrease in high energy phosphates and a fall in the intracellular pH.

All three phosphorus atoms of ATP are visible in ^{31}P MRS, with the α -resonance peak at -7.6 ppm, the β -peak at -16.3 ppm, and the γ -resonance at -2.6 ppm. Additional visible peaks are from phosphomonoesters (PME) at 6.8 ppm, phosphodiester (PDE) at 2.9 ppm, diphosphodiesters (DPDE) at -8.1 ppm, and uridine diphosphoglucose (UDPG) at -9.6 ppm. Present-day human cardiac MRS protocols¹⁶⁴ comprise significant inputs from ^{31}P MRS.

To illustrate typical application areas of ^{31}P MRS from our own studies, *in vivo* ^{31}P MRS of a post-poliomyelitis patient revealed abnormal phosphate metabolite ratios compared with normals¹⁶⁵. We have recently also reported¹⁶⁶ ^{31}P

MRS results on an experimental model of lymphatic filariasis which continues to be a major public health problem in tropical and subtropical countries

(v) ^{13}C MR Spectroscopy

Certain features of muscle (energy) metabolism are more suited for studies by ^{13}C MRS than ^{31}P MRS. Thus, ^{13}C MRS can provide access to key metabolites in muscle glycogen storage and glycolysis, the citric acid cycle¹⁶⁷ and in glycogen and fat utilisation¹⁶⁸ that are not accessible to ^{31}P MRS¹⁶⁹. A familiar example is provided by diabetes mellitus, a serious metabolic disease affecting tissues in which carbohydrate metabolism involves the sorbitol activation pathway

In a representative early application of double resonance techniques to *in vivo* clinical studies, Bottomley *et al.*¹⁷⁰ combined spatially localised, natural abundance ^{13}C MRS with ^1H -decoupling and nuclear Overhauser enhancement to improve ^{13}C sensitivity nearly five fold in the human leg, liver and heart. In another interesting application of double resonance spectroscopy¹⁷¹, the combined results of localised ^1H MRS and broadband proton-decoupled ^{31}P and ^{13}C MR spectroscopy on human tumour and skeletal muscle tissue have been used for the noninvasive observation of almost the complete pathway of phospholipid synthesis and degradation. The particular need for observing nuclei other than ^{31}P and ^1H in human cardiac MRS has been discussed¹⁶⁴.

12 Clinical MRI and MRS in Century Twenty-One: A Critique

During its relatively short but fast-growing tenure, MRI has quickly become a competitive, and often the only, modality in the wide and fascinating scenario of medical diagnostic imaging. Although the original important applications started in the area of neuroradiology (namely, the central nervous system comprising the brain and spine), the modality is now applied also to musculoskeletal, cardiac, abdominal and extremity studies. The existing facility in the Departmental laboratory is a 'whole-body' imager which is routinely used for all the above diagnostics.

As the Magnetic Resonance technology matures, the technological limits on the achievable scanning speed and pixel resolution are rapidly approaching

the limits imposed by the laws of physics, while the methodological limits of the technique are set by physiological safety considerations. For example, the achievable upper limits of gradient strengths ($G \approx 23\text{--}25\text{ mT/m}$) and gradient 'slew rates' (dG/dt) are restricted only by the important safety consideration of avoiding peripheral nerve stimulation in the patient by too rapid a gradient switching. Newer generations of commercial MRI magnets are likely to be more 'open' superconducting structures, both in order to minimise patient claustrophobia and to facilitate '*in situ*' interventional procedures by the clinician.

Regarding the role of MR spectroscopic techniques in routine clinical diagnostics, the growth of MRS to the fully clinical level has been somewhat stunted due to the several methodological complexities reviewed in earlier sections. Unlike MRI, which burgeoned very rapidly as a diagnostic tool thanks to prior radiological insights already gleaned from clinical X-ray computed tomography (CT), *in vivo* MRS explores a relatively vast and less well-chartered territory, and cannot use prior knowledge to define which spectroscopic fingerprints are relevant to the expression or definition of a disease. In the MRS of the living brain, in particular, routine and speedy clinical protocols have not yet been established to give it the status of a 'non-invasive biopsy'¹⁷². Rather, the information obtained continues to be novel and unexpected, such as the left-right asymmetry in the *in vivo* distribution of normal brain metabolites¹⁷³ and the regional variability of several brain metabolites in the white and grey matter areas, cerebellum and thalamus¹⁷⁴. What does constitute a giant step forward, however, is that simple biochemical markers of disease can, and do, become available noninvasively¹⁷⁵.

In the immediate future, at any rate, the bulk of the contemporary clinical MR spectroscopy will continue to comprise ^1H MRS. An important technical advantage in preferring ^1H MRS over ^{31}P MRS is that the signal from the former nucleus is 15 times more sensitive. This implies that a molecular group such as $-\text{CH}_3$ can effectively yield a 45-fold sensitivity increase. There are two ways of 'investing' this increased sensitivity. One is the obvious signal-to-noise enhancement, which can reduce scan time. The other, more important, benefit is an improved *spatial* resolution. This last factor is important in clinical spectroscopy since it

can lead to a 3-4 times reduction in the linear dimensions of voxels relative to ^{31}P MRS. This facilitates investigation of anatomically meaningful regions of the human brain, e.g., it would be of significant interest to be able to determine metabolites specific to glia, in much the same way that NAA is a specific neuronal marker. The glial specificity of scyllo-Inositol (an isomer of inositol) is a topic of current interest¹⁷⁶, in this respect, the assignment of a proton signal centred at 3.3 ppm to scyllo-Inositol¹⁷⁷ is of significance.

An important limitation of MRS ought to be pointed out in the context of the cell specificity of metabolite studies. To date, the resonances in the ^1H MR spectrum from a given anatomical region in the brain provide *composite* information about the different cell types and probably also about metabolic/neurological interactions¹⁷⁶. More *in vitro* MRS studies combined with biochemical analyses will be necessary to define *cell-specific* metabolites. Despite some ingenious experimental efforts in this direction using a guinea pig model¹⁷⁸, clinical MRS is unfortunately not yet ripe for detecting small peptides or neurotransmitters, and the metabolic heterogeneity at the cellular level.

The above limitations apart, the clinician of today has an excellent opportunity to use MRS for obtaining useful *in vivo* metabolic and neurochemical information¹⁵⁴ without the use of any tracer or radioactive molecule. For the presently employed techniques of MRS to be more putative in the clinical setting, the objective and reliable determination of *absolute* metabolite concentrations becomes very essential¹⁷⁴, and a number of approaches to the automated analysis of metabolite data have become available through spectral modelling methods^{179,180}. Information that accrues from such methods should hopefully lead to a 'molecular level' understanding of various

tissue pathologies, their progression, regression, and treatment. Also, future advances in *in vivo* MR technology will almost certainly be in the direction of facilitating the full integration of anatomical and functional MRI with metabolic and functional MRS. Early stage disease diagnosis and therapeutic strategies should make significant advances from such developments.

13 Summary

This review provides an exhaustive survey of the many basic principles and techniques of Nuclear Magnetic Resonance (NMR) which have been successfully exploited in the clinical setting for *in vivo* disease diagnosis. Various signal encoding methodologies and the signal intensity contrast mechanisms have been discussed, and a number of examples given of their applications to anatomical imaging (MRI) and functional imaging (fMRI). The concept of *k*-space, and the various fast techniques of image acquisition in *k*-space, have been documented. Magnetic Resonance Angiography (MRA) of flowing spins, the effects of diffusion and perfusion on MR images, and tissue contrast enhancement strategies using exogenous paramagnetic agents have also been described. Some recent MR imaging experiments involving hyperpolarised spins have been cited.

The latter part of the review deals in detail with several important single voxel and multi-voxel localisation techniques which enable *in vivo* MR spectroscopy (MRS). The focus of these discussions is the application potential of rest-state ^1H , ^{31}P and ^{13}C MRS and functional ^1H MRS (fMRS) in diagnostic medicine. It is concluded that future advances in *in vivo* MR technology will be in the direction of facilitating the full integration of anatomical and functional MRI with metabolic and functional MRS, which in turn will enable early stage disease diagnosis and therapy.

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APPLICATION OF NUCLEAR MAGNETIC RESONANCE IN AGRICULTURE

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In agricultural research, the major emphasis is given to analysis of large number of samples for various chemical constituents and physical properties. The method should be rapid and non-invasive especially in germplasm evaluation and plant breeding programs. Nuclear magnetic resonance technique which has the potential to meet both these demands has been extensively used in studies related to agricultural plants and their products. The principle of free induction decay (FID) of low field NMR is used for rapid and non-destructive determination of oil and moisture in oil seeds. Both spin-spin and spin-lattice relaxation times (T_1 & T_2) are exploited to obtain degree of saturation of oil in oil seeds and dry rubber content in natural rubber latex. They are extensively used to study water status and their cellular compartmentation in plant tissues and to develop a screening technique for drought tolerance in wheat. The application of high resolution NMR especially with ^{31}P and ^{13}C nuclei are also quite substantial. The fatty acid composition of oil in a single intact seed is obtained using ^{13}C NMR. Phosphorus NMR is extensively used to elucidate the mechanism of phosphate uptake and compartmentation in roots, anoxia in developing seeds and adaptation of roots to osmotic stress. The application of proton NMR imaging in studying the *in vivo* changes in water status in stems is also explored. Thus the review covers in detail the work carried out by our group using NMR techniques in characterising and quantifying plant traits of agriculturally important crops.

Key Words: Non-Invasive; Spin Lattice Relaxation Time T_1 ; Spin-Spin Relaxation Time T_2 ; Tissue Water Status; Cellular Compartmentation; Drought Tolerance; Phosphate Metabolism; Proton NMR Imaging

1 Introduction

NMR is a powerful and versatile technique with ever increasing range of applications in almost all disciplines of science. Unlike many analytical techniques that are able to determine just one material property, NMR techniques are unique in their ability to monitor several essential material properties related to both structure and dynamics. It is a relatively insensitive technique (less sensitive than other analytical techniques) but its non-invasive feature provides the incentive to exploit the method. The non-invasive nature of the measurement makes it possible to obtain the information *in vivo* which has several advantages over conventional analytical techniques. Agricultural and food samples are complex heterogeneous structures composed of many different components like water, fat, protein, carbohydrates and flavours etc. The characteristics

of most material including the storage and textural properties are related to how these various components interact.

The latest technological innovations and advances in high resolution multi-nuclear NMR instrumentation including higher magnetic field, large sample probe heads, fast computing devices, new pulse sequences and capability to record solid state spectra through CP (Cross Polarization) and MAS (Magic Angle Spinning) and possibilities to monitor low abundant and less sensitive nuclei, have made the application of this technique increasingly interesting. These applications are being actively pursued in many laboratories for analysis of biological samples, specially animal and human tissues. However, in plant tissues the application of this technique has been relatively limited.

The wide range of application of NMR in food

and agriculture can be divided (based on types of instrumentation available) into following groups;

- 1 Low Resolution/Low Field NMR
- 2 High Resolution NMR Spectroscopy
 - (a) Solution state (b) Solid state (c) *In-vivo*
- 3 NMR Imaging

A few typical examples are given below.

1.1 Low Resolution NMR

(Elucidation of physical structure from analysis of NMR decay signal)

- Non-destructive analysis for:
- Solid content in fat/oil blends
- Moisture and/or fat content of food products.
- Determination of oil content in intact oil seeds
- Determination of economically important parameters of plant and dairy products

(Relaxation times T_1 & T_2 which provide information about molecular mobility are unique for probing micro dynamics in food or plant systems)

- Water relationship in plants and food products
- Interactions of water with macromolecular structure

Pulsed field gradient NMR – (Diffusion coefficient measurement)

- Micro heterogeneity studies of food matrix (e.g. measuring the size of water droplets in emulsion or complex structures)

1.2 Solution state High Resolution NMR

- Elucidation of chemical structure of extracts
- Fatty acid composition of intact oil seed
- SNIF – Site specific Natural Isotopic Fractionation NMR

(Authenticity criteria for a variety of food products)

^2H -NMR-Characterization of concentrated fruit juices, estimation of age of spirits or dating of alcoholic beverages etc.

1.3 Solid state High Resolution NMR

CPMAS – Cross Polarization Magic Angle Spinning for

- Elucidation of chemical structure
- Information on the physical structure of solid material

- Protein analysis of intact seeds
- Characterization of soil humic and fulvic substances, and aromaticity of soil organic matter

1.4 *In vivo* NMR Spectroscopy

Relationship between metabolism and physiological activity is not fully known. Non-invasive technique can provide metabolic information that would be lost on extraction.

In vivo ^{31}P NMR provides a window for measuring:

- Intracellular pH – (expensive pH meter)
- Intracellular compartmentation of ions and metabolites (continuous and simultaneous monitoring)
- Metabolic pathways, rates of transport or reaction
- Understanding energy metabolism and metabolic traits associated with hypoxia induced injury ^{13}C , ^{14}N , ^{15}N NMR
- Monitoring plant metabolism, intracellular uptake of NO_3^- and NH_4^+ ions; ^{23}Na , ^{39}K NMR (using shift reagent, intra & extra cellular sodium or potassium can be detected)

How various plants survive in saline environment can be elegantly studied.

1.5 NMR Imaging:

The potential applications of MRI are fascinating and numerous.

Non-invasive observation of

- Development of fungal infection in fruit
- Various quality factors in fruits or vegetables
- Evaluation of internal quality parameters such as bruises, dry regions, worm damage, stage of maturity and presence of voids.
- Hydration of wheat and dry grains-the extent of penetration of water (detection of dormancy).
- Temperature inside solid foods during heating and cooling procedures.
- Effect of freezing and thawing of meat.

2 Low Resolution NMR

The application of low-field NMR have been relatively more exploited because of less expensive and simple instrumentation. One of the main

requirements in agricultural research is to analyze large number of samples for their one or more chemical constituents and physical properties. In plant-breeding programmes and germplasm evaluation, it is desirable that the samples are not destroyed in the process of analysis so that the identified genetic materials remain viable for further propagation. Pulsed Nuclear Magnetic Resonance (NMR) is a potential means for developing methods of such analysis.

Various applications of both low and high resolution NMR studied by our group are discussed in the following sections.

2.1 Development of Pulsed NMR Method for Rapid and Non-Destructive Determination of Oil Content in Oilseeds

One of the major constraints to develop high oil yielding varieties of oilseeds has been the lack of a rapid and non-destructive method for the determination of oil content in oilseeds. There are several well known advantages of NMR method of determination of oil content in comparison to the slow and destructive chemical methods of analysis.

Among the NMR methods, pulsed NMR is superior to the wide-line NMR method of oil determination in terms of elimination of angular orientation, requirement of magnetic field homogeneity and oil determination without weighing the seeds which makes the analysis much faster.

In oilseeds, hydrogen which gives strongest NMR signal is present mainly in four forms. oil, moisture, carbohydrate and proteins. Owing to the different mobilities of the ^1H nuclei in these constituents, their spin-spin relaxation times (T_2) are quite different. The T_2 values in protein-carbohydrates, moisture and oil are of the order of microseconds, milliseconds and hundreds of milliseconds respectively. The decay of NMR signal in oilseeds thus consists of a rapidly decaying signal from the solid component (protein-carbohydrate) and slowly decaying signal from the moisture and oil. The basic principle in determination of oil content in oilseeds is to measure the NMR signal of oil alone and compare it with the signal in a known amount of pure oil under identical experimental conditions.

The pulsed NMR method using liquid signal for

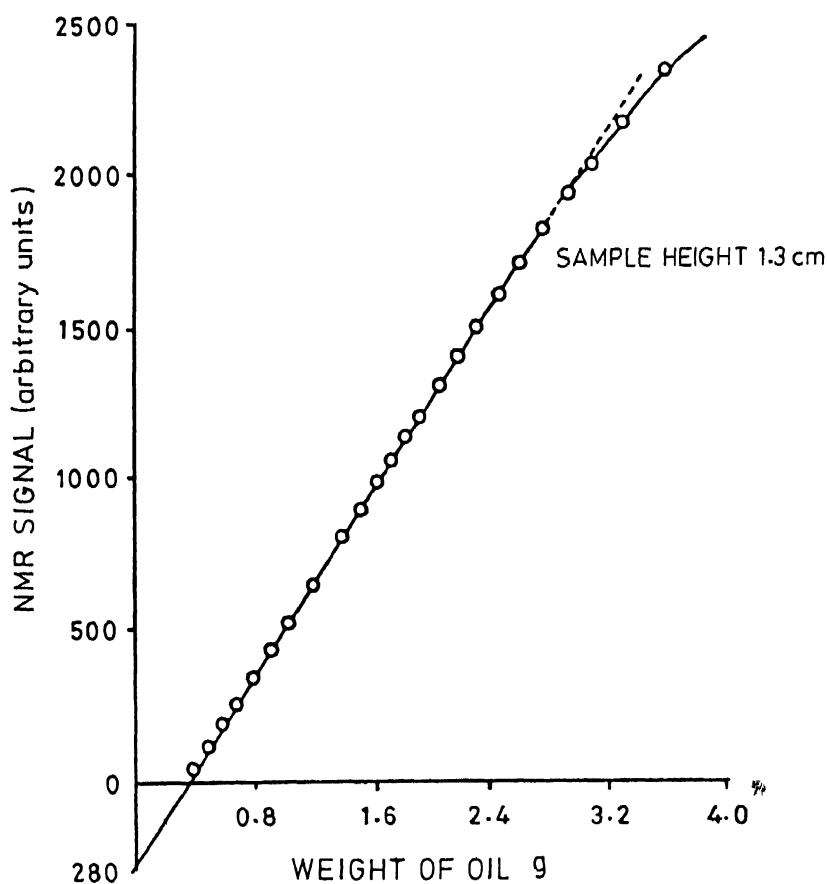


Fig. 1 A typical calibration curve for pure mustard oil using the FID signal

seed oil determination was developed at NRL in the early 1970s after careful study of the various parameters affecting the NMR signal, the possible sources of error and the corrective steps required to obtain reproducible and reliable results¹. The parameters studied included the effects of T_1 and T_2 , seed moisture, the angular position of the seed, sample tube thickness, sample height and reproducibility of the NMR signal. The effect of sample to sample variations in T_1 on the NMR signal can be eliminated by fixing the pulse repetition time long enough (five times the longest anticipated T_1 value) to attain the full equilibrium magnetization. A pulse repetition time of 1s is sufficient, as the T_1 of most oilseeds is usually less than 200 ms. The decay of oil signal after 90° pulse is generally independent of sample to sample variation in T_2 of the oil as it is governed by T_2^* which depends upon the magnetic field inhomogeneity of the spectrometer used for such work.

The interference of moisture in oil signal and effects of seed orientation on the FID signal due to magnetic field inhomogeneity can be eliminated by optimal choice of sampling delay times. The seeds of sunflower, peanut and mustard are dried (usually overnight at 50-60°C) to reduce the moisture content to 5% and FID signal is sampled at a delay time of 250 μ s. To determine the oil content of any oilseed crop, suitable parameters are chosen and a calibration curve (Fig.1) is constructed by recording the NMR signal of known increasing quantity of pure oil extracted from oil seeds of the same crop grown under a similar environment to that being tested. The quantity of oil in an unknown seed sample can be read against its NMR signal

from the calibration curve. Using this method the oil contents of mustard, peanut and sunflower seeds have been determined and compared with those by chemical extraction methods (Table I). The values obtained by NMR not only agree with that by extraction method but the reproducibility of the NMR method is better than the standard extraction method.

2.2 Rapid and Non Destructive Determination of Oil in Oil Seeds, without Weighing and Drying the Seeds

(1) FID Signal Method

Though the liquid signal method of seed oil determination as described above is quite accurate, the seeds are required to be dried and weighed, which makes the analysis considerably slow and not truly non-destructive. To overcome these limitations, the solid-liquid signal method was developed which does not require either weighing or drying of seeds². The method is based on the measurement of FID signals of protons of solid and liquid constituents of mustard seeds (Fig. 2). The signal measured around 10 μ s (immediately after the dead time of the receiver) is contributed full by liquid phase signal (L) and partly decayed solid phase signal (S') in the seeds. The signal measured at 70 μ s represents nearly full liquid phase signal only as solid-phase signal has almost completely decayed by then. For samples having similar moisture and solid phase T_2 , the oil content is calculated as:

$$\text{Seed oil content (\%)} = \frac{L}{L + fS'} \times 100$$

Table I

Comparison of Pulsed NMR (Liquid signal FID) and chemical (Cold Percolation) methods for oil determination in mustard, peanut and sunflower seeds

Sample No.	Crop Method	Oil Percentage					
		Mustard		Sunflower		Peanut	
		NMR	Chemical	NMR	Chemical	NMR	Chemical
1		48.3	47.3	45.3	46.0	49.5	48.8
2		47.0	45.1	39.3	41.9	50.1	49.1
3		45.9	44.4	44.7	42.4	49.9	51.1
4		43.1	42.3	42.0	41.2	50.7	48.3
5		43.5	43.2	41.8	41.3	47.2	46.3
6		45.4	45.3	35.6	37.3	49.8	51.1
7		29.3	29.1	33.3	34.1	43.4	44.4
8		46.1	46.0	36.7	36.8	47.4	47.9
9		40.0	38.8	45.2	45.6	46.9	46.9
10		48.5	48.5	37.6	36.4	46.8	47.6

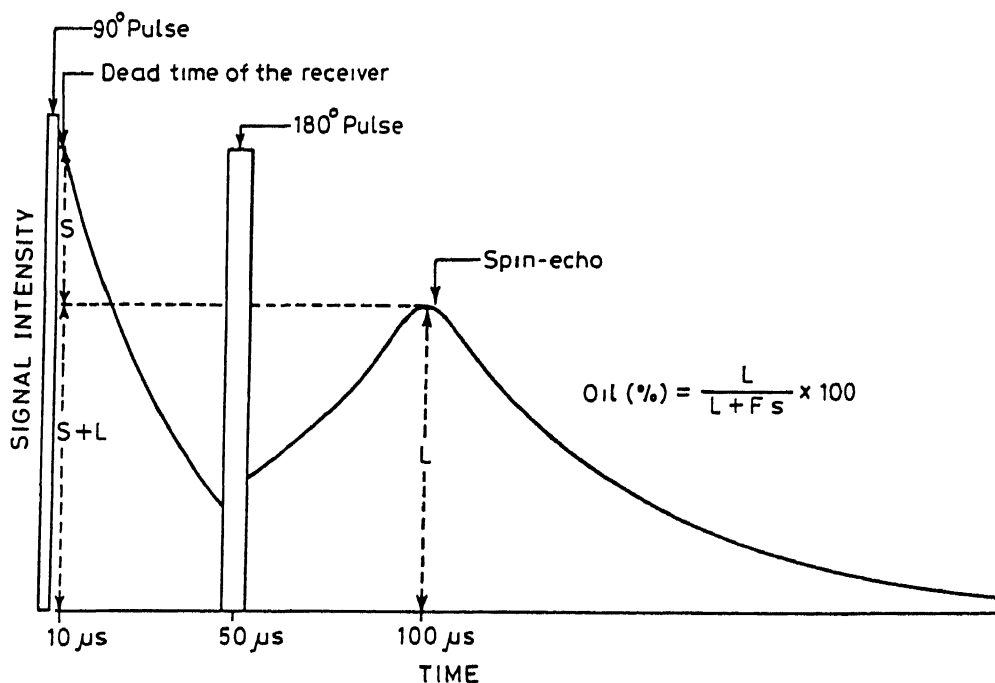


Fig 2 FID signal of the same mustard seeds dried in sun and at 105°C

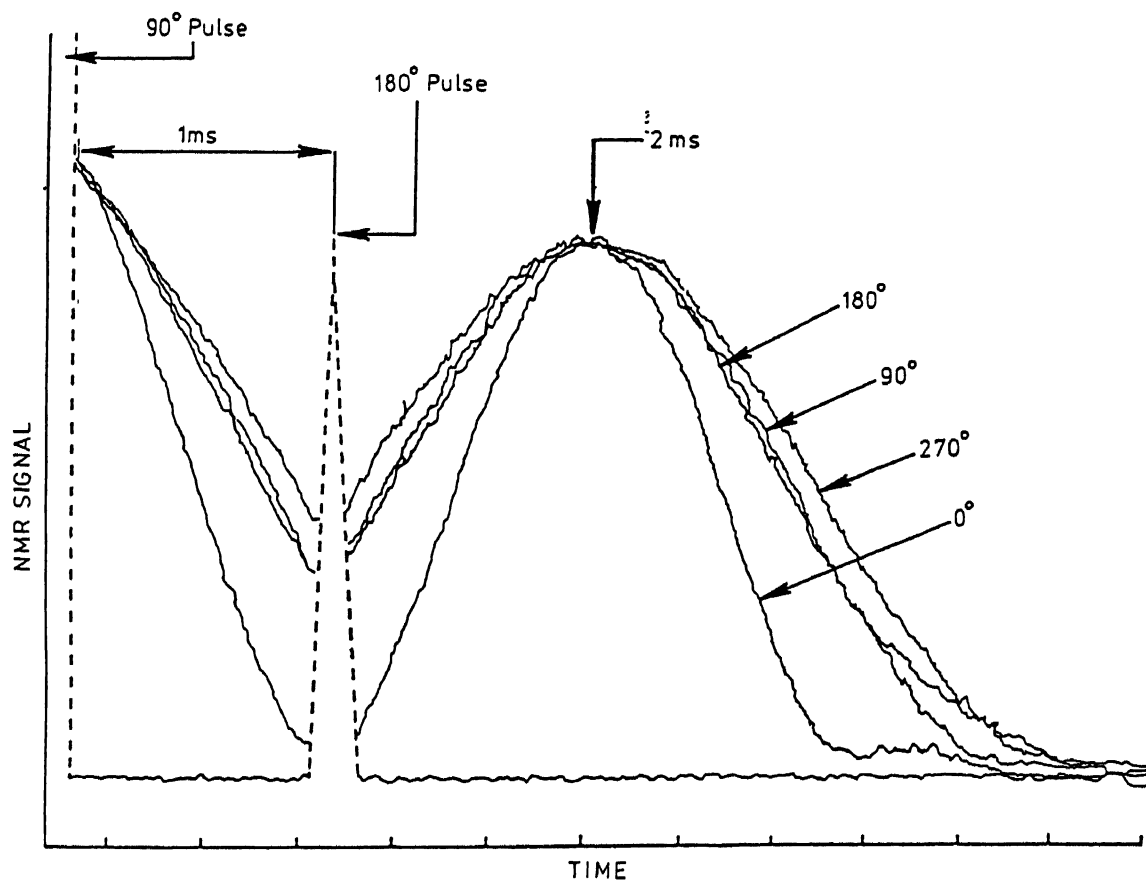


Fig 3 Variation in free induction decay and spin-echo signal of the same single peanut seed at different angular orientations. The initial position of the seed in the nuclear magnetic resonance (NMR) tube was taken arbitrarily as 0° , and the tube was rotated by 90° each time.

The correction factor f accounts for the decay of solid signal during the dead time of the receiver, proton density difference in solid and liquid phases of oilseeds and the residual moisture signal at $70\mu\text{s}$. It has been shown using mean " f " values calculated from the above equation, using sun dried samples of mustard, sunflower and soybean seeds of known oil content, that oil content in seeds can be determined without weighing or drying¹. The accuracy of measurement by solid-liquid signal method is good and the method is suitable for the large scale screening of oil seeds required for efficient implementation of plant breeding programmes aimed at developing high oil yielding varieties of oil crops.

(ii) *Spin-echo Signal Method (using Low Homogeneity Magnetic Field)*

The free induction decay (FID) and echo signals in a spin-echo pulse sequence have been used for seed oil determination without weighing and drying the seeds³. The method overcomes the problems

caused by low homogeneity of the magnetic field. The earlier known method, based on use of the FID signal to determine seed oil, becomes inapplicable when the magnetic field homogeneity is poor, because the angular position of seed affects the signal significantly. The present method which elegantly eliminates the angular dependence (Fig 3), involves sampling the FID signal at $10\mu\text{s}$ after a 90° pulse and the subsequent echo signal at $100\mu\text{s}$ formed by applying 180° pulse at $50\mu\text{s}$ (Fig. 4). Such a short pulse spacing in spin-echo sequence produces an almost full oil signal. It also eliminates the effects of sample-to-sample variation in T_2 and molecular diffusion. The oil values obtained by this method are in good agreement (Table II, correlation for mustard: 0.952, linseed: 0.99; and for peanut: 0.912) with the values obtained by the well established and accurate pulsed NMR method, which is based on the measurement of the FID signal of oil in dried and weighed seeds.

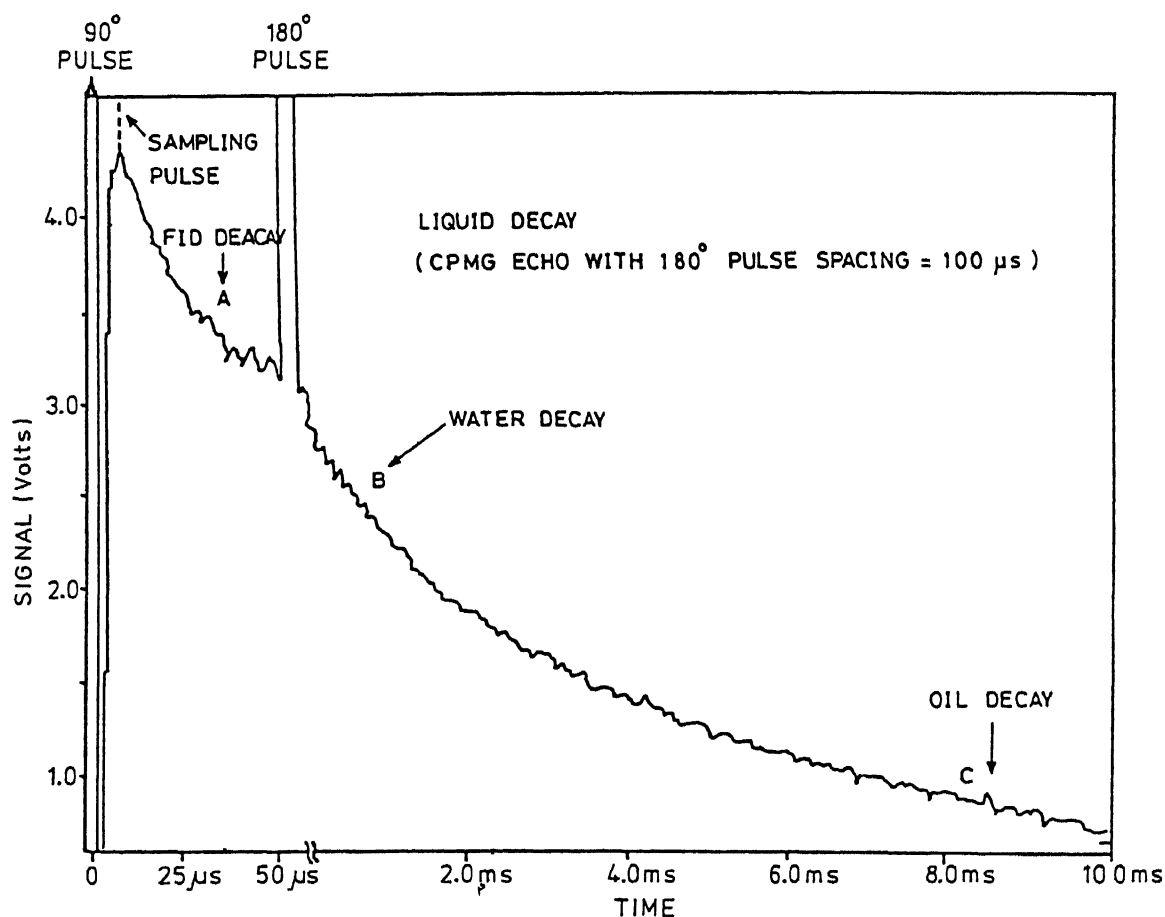


Fig 4 The schematic diagram of the spin-echo pulse sequence used in the present work for seed oil determination without weighing and drying the seeds. The full liquid signal (L) and partly decayed solid signal (s) are shown by arrows.

Table II*Comparison of oil content (%) measured by the present method and the earlier reported free induction decay (FID) method*

Sample No	Mustard		Linseed		Peanut	
	Present Method	FID Method	Present Method	FID Method	Present Method	FID Method
1	42.6	42.7	43.0	42.8	51.7	51.5
2	40.5	41.0	47.3	47.0	53.6	54.2
3	41.6	41.1	38.1	37.9	48.3	50.4
4	38.9	38.5	43.8	43.3	49.2	49.8
5	40.5	41.1	43.1	42.6	52.2	52.5
6	38.7	38.2	45.3	44.8	48.7	48.8
7	39.6	39.3	42.7	42.0	54.7	53.8
8	40.1	39.6	43.4	43.9	51.8	50.4
9	40.4	40.0	44.3	43.8	54.5	54.8
10	39.8	39.0	—	—	52.9	53.7

2.3 Simultaneous Determination of Oil and Moisture Content in Oilseeds

The variation of proton relaxation time (T_2) has been studied for paddy seeds having different levels of moisture. The spin-echo signal has been differentially resolved into moisture and oil components^{4,5}. The change in T_2 with moisture content has been used as method of moisture determination in paddy and a calibration has been worked out⁴. The differential relaxation behaviour of protons in solid constituents, water and oil has been used for simultaneous determination of oil and moisture in mustard seeds using CPMG pulse sequence⁶. This method is based upon:

Resolving the CPMG echo signals into exponentially decaying liquid phase signals (two components due to moisture and one component due to oil). Sampling FID signal following 90° pulse in CPMG sequence at $t = 10\mu s$ for determining signal contributed by protons in the solid matrix of seed.

Computation of moisture and oil content using respective extrapolated signals, extrapolation coefficient for solid component decay and correction factor for proton density differences in water, oil and solid constituents of seeds.

The moisture and oil content thus determined simultaneously and without weighing the seeds have been found to be in good agreement with moisture content determined gravimetrically and oil content determined by the liquid signal FID method⁶.

The choice of a suitable pulse sequence depends upon the purpose of the determination. The various methods of pulse NMR technique for seed oil

determination have been reviewed in a recent article⁷.

2.4 Application of NMR Method of Oil Determination in Oilseeds Research

2.4.1 Effect of Maturity on oil Yield of Mustard Varieties

In a study on oil bio-synthesis, it has been found that in Brassica *Juncea* var. Varuna the oil percentage reached its highest value at 55 days after flowering, whereas 100 seed weight was highest about 20 days later, when the pods were fully yellow⁸. This information shows that the crop should be harvested at the yellow podded stage for maximum oil yield. In another experiment with six mustard varieties, it was found that oil yield was optimum at yellowish brown podded stage in almost all the varieties which suggests that mustard should be harvested when the pods are nearly brownish yellow as seed yield would be lower if crop was harvested earlier⁹. At later stages there would be loss of seeds due to shattering.

2.4.2 Mass Screening for Improving Oil Yield of Mustard

In collaboration with oilseed breeders, the inter-varietal and intra-varietal variability in oil content of several cultivated mustard variety have been studied using pulsed NMR. A large plant to plant variation in oil content (30 to 50 percent) was found in most of the varieties¹⁰. Selecting seeds with higher oil content and further propagating them for two years, appreciably increased the oil content without any decrease in seed yield (Fig. 5). The increase in oil yield was as high as 20% in a yellow sarson variety. Using this simple selection technique, the existing rapeseed, mustard varieties

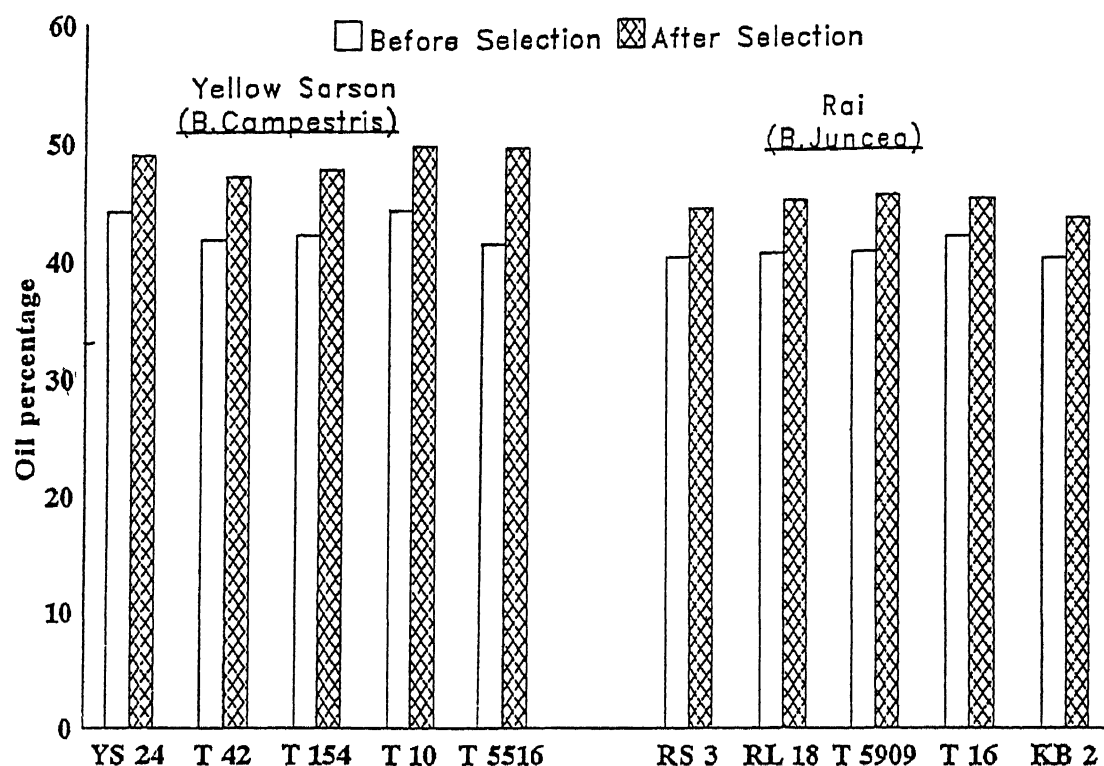


Fig 5 Increase in oil content of mustard varieties after two years of mass selection of high oil content using pulsed NMR

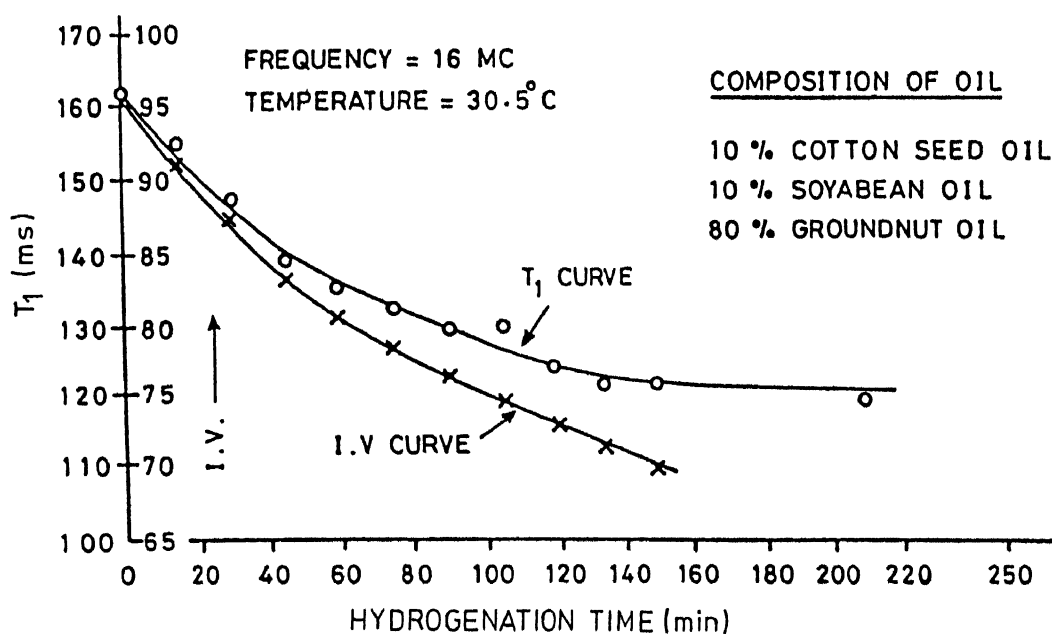


Fig 6 Variation in spin-lattice time T_1 and Iodine value of oils with its hydrogenation time.

and perhaps other oil crops can be improved for giving higher oil yield without adding any extra inputs.

2.4.3. Effect of Rhizobium inoculation on Oil Content of Soybean Seeds

It is well known that oil percent of soybean

decreases with an increase in yield when inoculated with Rhizobium strains. This was confirmed by two independent experiments using strains of *R. Japonicum* and their composites. These experiments also showed that the initial decrease in oil content over the controls stabilized at the reduced value and the seed yield continuously

increased with time resulting in higher oil yield in the inoculated plants¹¹.

2.4.4. Oil and Protein Synthesis During Grain Development in Sunflower

Our study on a sunflower variety EC 68414 showed that oil percentage reached its highest value on 23rd day after flowering, thereafter the differences were marginal. The physiological maturity for oil yield was however attained at 33 DAF when leaves and capitulum were yellowish brown, seed dry weight was highest and moisture content was reduced to about 36%. For protein productivity, the physiological maturity was observed a little earlier, viz 23 DAF. The study should enable the crop to be harvested at proper stage so as to escape diseases and pests attack¹².

2.5 Estimation of Degree of Saturation of Oil in Oil Seeds

The degree of unsaturation of oil (measured as Iodine value) is an important parameter of its quality in terms of the nutritional value. It takes about 5 to 6 hrs to measure the Iodine value including the time taken for extracting the oil. Though high resolution ¹³C FT-NMR has been reported in the literature to estimate the relative proportion of unsaturated and saturated fatty acids, it may not be suitable for large scale screening because of very expensive spectrometer and long analysis time required for it. However, we have found the T_1 of oil protons, which can be measured non-destructively, using low resolution NMR changes in a similar way as the iodine value except in the later stages of hydrogenation, when the oil starts changing to semi-solid and T_1 becomes insensitive to changes in viscosity (Fig. 6). It may be inferred that as long as oil is in the liquid form T_1 may give an estimate of the degree of unsaturation¹³. The plot of T_1 vs the I.V. for most of edible oil is a straight line (Fig. 7.), and the behaviour of oils that deviate from this line has been attributed to large differences in their molecular weights (saponification values). Thus the degree of unsaturation of edible oils whose molecular weights are similar to those used for calibration can be measured by pulsed NMR method in nearly 2 minutes using their T_1 values.

2.6 Rapid Determination of Dry Rubber Content in Natural Rubber Latex

Efforts are being made to develop a rapid and

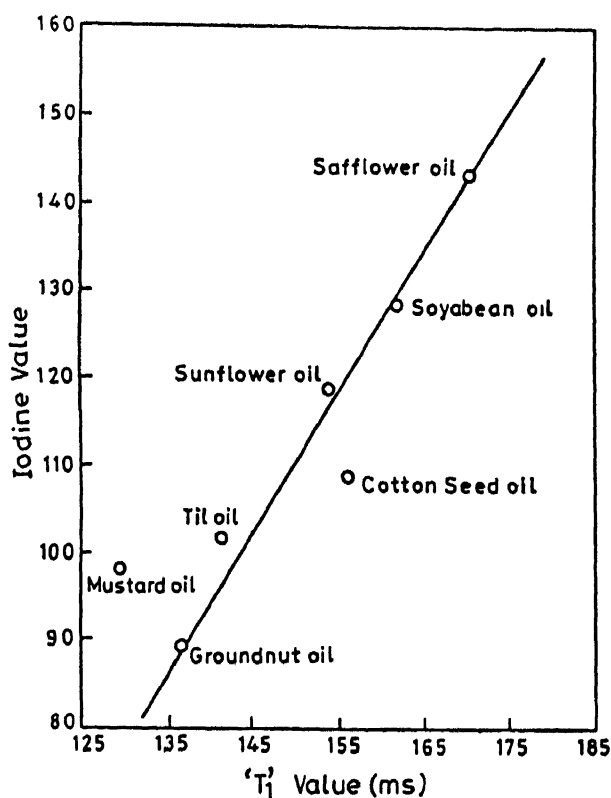


Fig 7 Iodine value of different seed oils plotted against their spin-lattice relaxation time

precise method of determining DRC for speedy marketing of rubber latex and for evaluation of agronomic practices on rubber yield of rubber plants. Such a method has been developed through the use of Pulsed NMR in collaboration with the Rubber Research Institute, Kottayam¹⁴. The method is based on the fact that the relaxation rates of solid and aqueous phases of the latex are markedly different. In this method the NMR spin-echo technique has been used to resolve the signals contributed by the rubber particles (mostly solid) and the aqueous phase of latex, since the signal can not be resolved by FID method where both phases practically decay with T_2^* . The DRC values obtained by the method are in good agreement with those obtained by the standard laboratory method as shown in Table III ($R^2=0.991$, $P=0.001$). The study shows the feasibility of a quick and precise NMR method of DRC determination without requiring any preparation or weighing of sample or a calibration curve.

2.7 Estimation of Water Status in Crop Plants

Water plays an important role in all physiological processes in the plant and its deficit

has long been known to affect almost all aspects of plant growth and metabolism. Characterization of water status and its availability in plant tissues assumes importance in studies where water becomes limiting. Pulse NMR measurements of longitudinal relaxation time (T_1) and transverse relaxation time (T_2) have been used to study water status and its interactions in biological systems as

Table III
Comparison of pulsed NMR and conventional methods of determination of dry rubber content in rubber latex

Sample No.	Dry Rubber Content (%)	
	SLM	NMR
1	14.9	15.6
2	26.1	26.6
3	26.1	26.1
4	32.1	32.2
5	32.4	32.9
6	34.8	34.7
7	35.0	35.0
8	35.5	35.6
9	36.1	36.7
10	35.5	36.2
11	36.5	35.6
12	39.5	41.5
13	39.3	39.5
14	39.8	40.5
15	40.4	41.8
16	41.5	42.8
17	42.6	43.7
18	43.7	43.7
19	44.0	45.6
20	46.5	45.9

SLM—Standard Laboratory Method

they directly relate to translational and rotational mobility of water molecules in homogeneous systems. However, in heterogeneous systems like leaf tissues, the mobility of water molecules gets modified and the relaxation time T_1 is influenced by macroscopic and microscopic distribution of water in different sites, macromolecular-water interactions and diversity of structure and size of plant cells. In matured leaf tissues, the vacuolar water occupies about 80% of the cell volume, with the cytoplasm forced by the turgor pressure into a thin layer against the cell wall. Therefore, T_1 of the mature leaf is dominated by the bulk water in vacuolar fraction and is a reflection of translational and rotational motion of leaf water. Higher relaxation time implies greater randomness of water molecules and better availability for participation in metabolic activities suggesting that leaf water T_1 which can be easily measured by pulsed NMR may be a good parameter for assessing water status in plants.

Experiments were conducted to establish the relationship of leaf water T_1 with other conventional physiological water status parameters to verify the above inference¹⁵. In two wheat cultivars, leaf water T_1 was found to have a logarithmic relation with both leaf water content (LWC) and leaf water potential (ψ) and a linear relation with relative water content (RWC) (Fig 8a-c). At similar LWC, ψ and RWC, the relatively drought tolerant cultivar IWP-72 maintained higher T_1 as compared to the susceptible cultivar HD

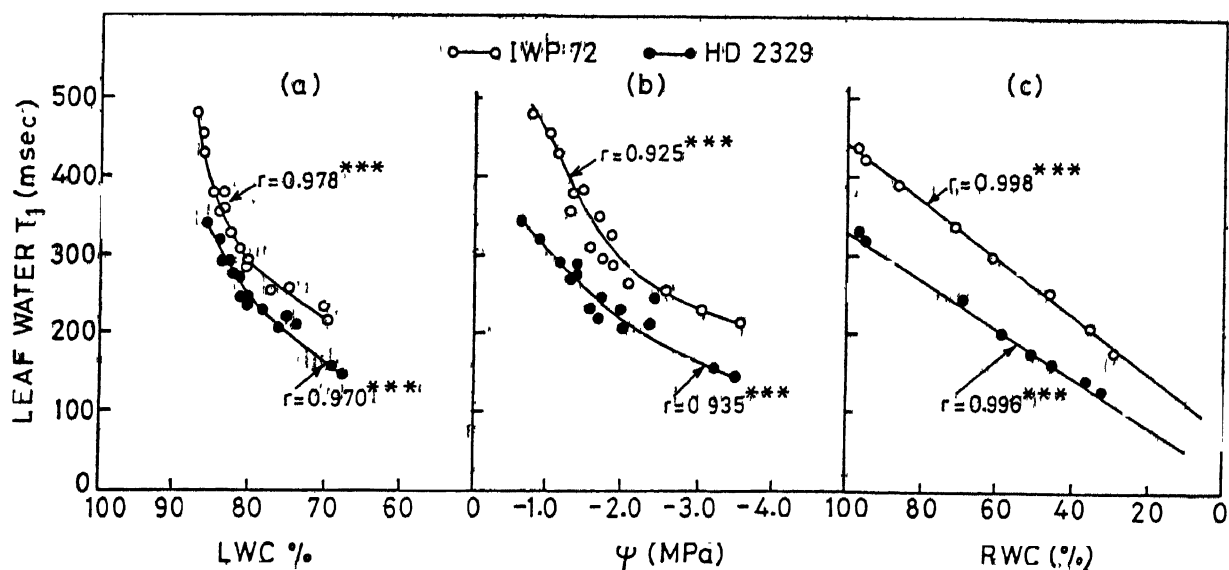


Fig. 8 Relationship of leaf water T_1 with (a) leaf water content, (b) leaf water potential and (c) relative water content in two wheat cultivars

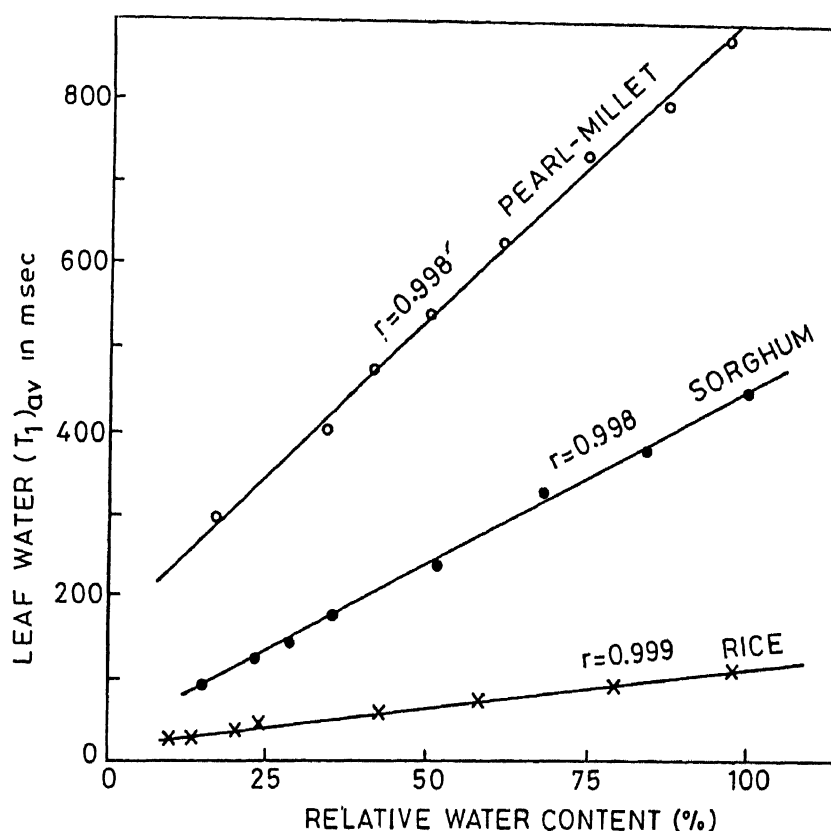


Fig 9 Relationship between leaf water T_1 and relative water content in excised leaves of three cereal crops

2329. This suggests that a drought tolerant cultivar is able to sustain higher metabolic activity than the susceptible at similar stress conditions. Similarly, a linear relationship between T_1 and RWC was obtained in the three kharif cereals viz. pearl millet, sorghum and rice in a dehydration experiment using excised leaves (Fig. 9).

After establishing the relationship of leaf water T_1 with conventional water relation variables, the superiority of leaf water T_1 over the other variables in describing the changes in plant water status was demonstrated in a pot culture experiment¹⁶. Plants of two drought tolerant cultivars (C-306 & IWP-72) and one susceptible cultivar (HD 2329) of wheat were raised under three different moisture regimes and changes in leaf water T_1 and other physiological water status variables were monitored from 45 to 75 days after sowing at weekly intervals. The three cultivars did not differ significantly in their values of LWC ψ and its components in the stressed and unstressed plants; but they differed significantly in their T_1 values under both conditions on all days of measurement

(Fig. 10). The drought tolerant cultivars C-306 and IWP-72 showed higher T_1 than the susceptible cultivar HD-2329 at all sampling dates in both conditions. This suggests that leaf water T_1 is a better parameter for describing plant water status than the traditional water relation indices.

An interesting observation in the experiment using three wheat cultivars grown at different soil moisture regimes¹⁵ was the significant logarithmic relation between ψ and T_1 (Fig. 8). ψ is also related logarithmically with water activity (a_w) which characterizes the state of water in the system by its availability for physical, chemical and biological processes, by the equation

$$\psi = RT/V \ln a_w$$

where R is the universal gas constant, T is the temperature in Kelvin and V is the partial molar volume of water. The logarithmic relation of ψ with both a_w and T_1 indicates that T_1 is linearly related to water activity and hence a better parameter than the conventional variables for describing plant water status.

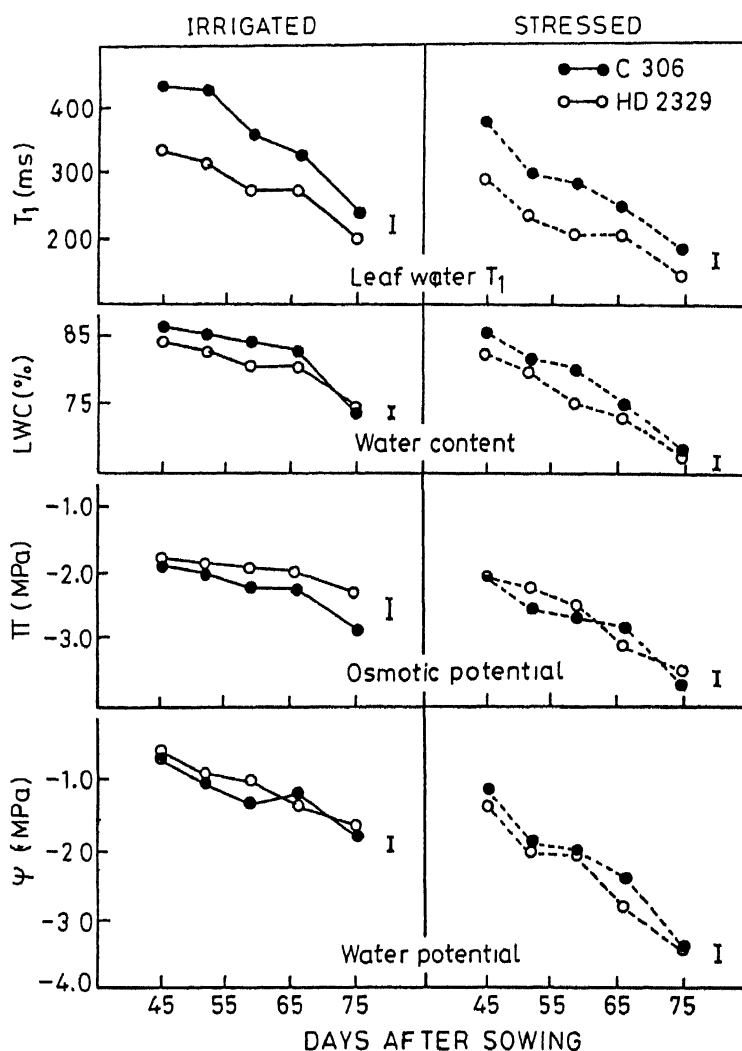


Fig 10 Changes in leaf water T_1 , leaf water content, water potential and osmotic potential of two wheat cultivars with maturity.

In order to test this hypothesis that T_1 is linearly related to water activity, an experiment was conducted in excised leaves of pearl millet¹⁷. Various levels of water activity were created using PEG-6000 solutions and the leaves were equilibrated. Measurements of LWC, RWC, ψ and T_1 were made and related with the imposed a_w values. With decreasing a_w , both RWC and T_1 decreased linearly and other variables exponentially up to a dehydration level of 0.978 below which there was abrupt reductions in all the parameters except T_1 which registered an increase (Fig. 11) The probable reason for such a sudden change can be attributed to the disruption of hydration layers of macromolecule which results in the release of water held in hydration shells and denaturation of macromolecules.

The state of water in biological tissues is significantly different from that of bulk water mainly because of macromolecular-water interactions which restricts its rotational and translational mobility. As the NMR relaxation times T_1 and T_2 measure the molecular mobility, they would be affected by tissue water content and several other factors like macromolecules and many solutes. Apart from tissue water content, which has been shown to be directly correlated with T_1 , other cell constituents like sugar, soluble protein and paramagnetic ions were reported to affect relaxation time in animal tissues. Therefore, these parameters were measured in cereal crops with wide variation in their T_1 values to explain the observed differences. It could be partly explained on the basis of their leaf moisture, soluble protein

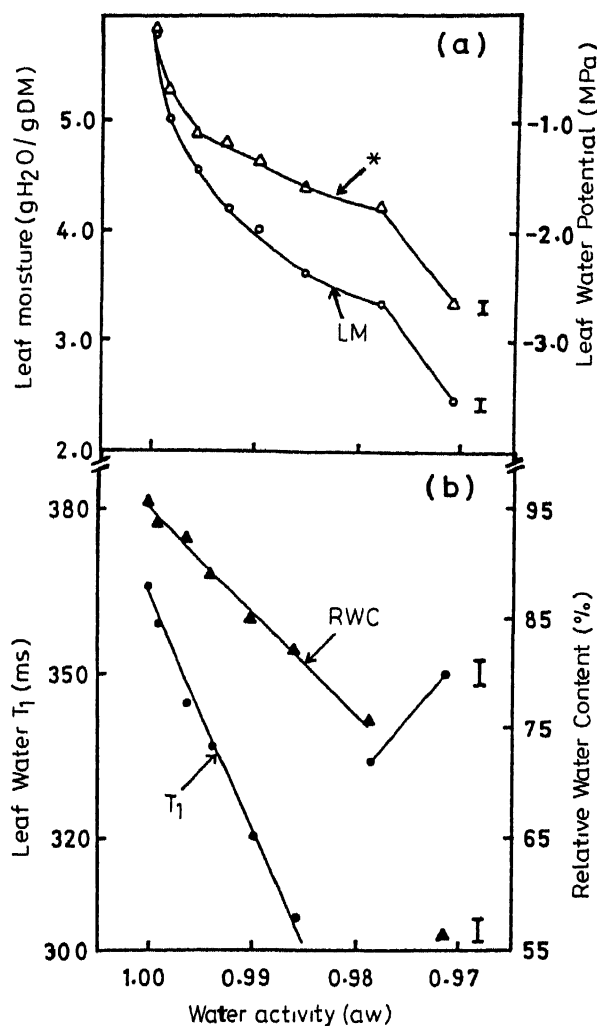


Fig. 11 Relationship of imposed water activity with (a) leaf moisture (LM) and leaf water potential (ψ), (b) leaf water T_1 and relative water content (RWC) in excised leaves of pearl millet

and soluble sugar in the cereal crops pearl-millet, sorghum, maize and rice¹⁸. The simple linear correlation coefficient of leaf water T_1 , with different cell constituents are given in Table IV. Water content was positively correlated and all other constituents were negatively correlated. Soluble protein and sugar showed highly significant relation with T_1 of leaf tissues.

The effect of soluble protein on relaxation time at different stages of growth was studied in barley and wheat leaves by maintaining the plants under different N-fertilizer levels^{19,20,21,22}. In both the crops, leaf moisture did not vary significantly among the control and N-fertilized plants. But protein content in leaves varied from the beginning of the treatment and its effect on T_1 was observed only after flowering stage of the crop (Figs. 12 and

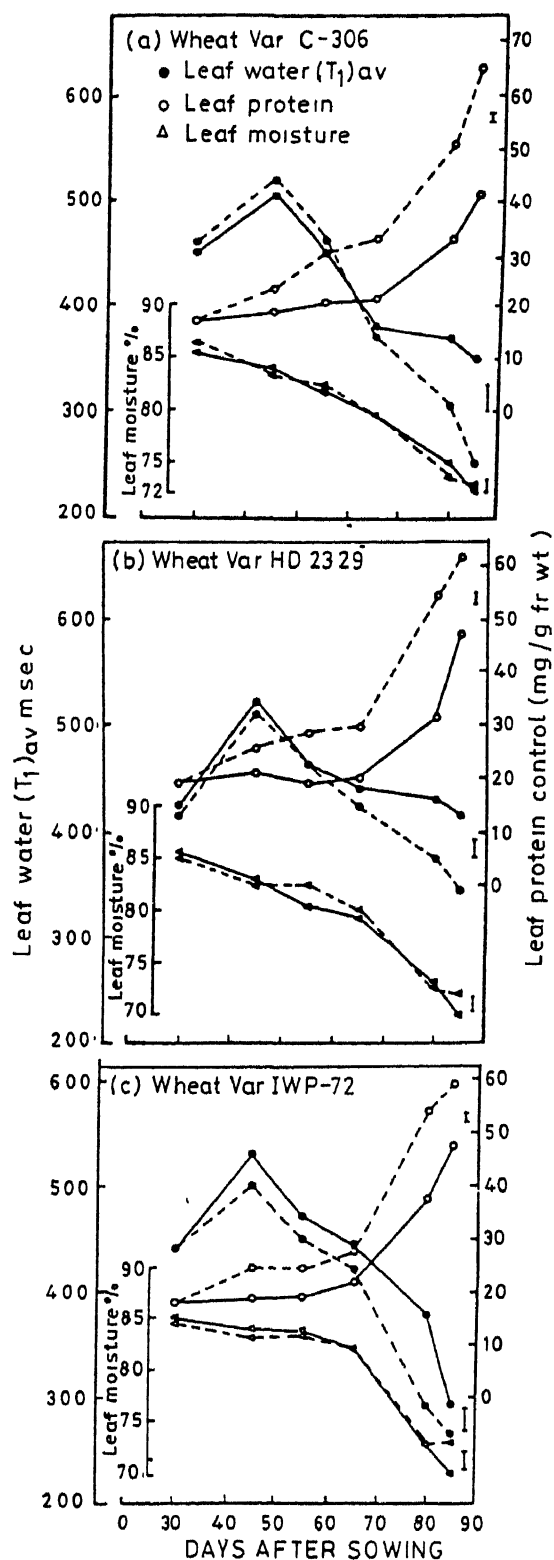


Fig. 12 Leaf water spin-lattice relaxation time T_1 , leaf moisture and leaf protein of wheat varieties (a) C 306, (b) HD 2329 and (c) IWP 72 at different stages of growth under two fertilizer treatments. Solid lines represent control and dotted lines represent N-fertilized plants. Vertical bars give LSD (at 5%) of corresponding parameters

13). This anomaly can be explained as follows: at early stages of growth there is an accumulation of sugar in N-deficient plants which equalled the reduction in T_1 due to enhanced protein content in N-fertilized plants. At later stages, there is no accumulation of sugar in N-deficient plants and a marked reduction in T_1 in N-fertilized plants was due to increased levels of leaf protein

NMR relaxation offers a number of advantages over classical techniques for determining water status as it determines not only internal water availability but also its physiological compartmentation, its states, and their variations under stress. Additional information could be obtained about cell water status by analyzing the dependence of the two components of spin-lattice relaxation time T_1 on protein and dry matter content in leaves of wheat and barley cultivars²³. The study confirmed the existence of at least two populations of water in leaves of wheat and barley with different relaxation characteristics. The relaxation rates of the two fractions were linearly related to both dry matter and protein contents (Fig. 14a & b). The longer and shorter components of T_1 for zero dry matter and for zero protein were calculated by extrapolation (Table V). From these

results it can be inferred that the fraction of water with short relaxation time probably is the water surrounding the cell wall and that of macro molecules and the other fraction with long T_1 is

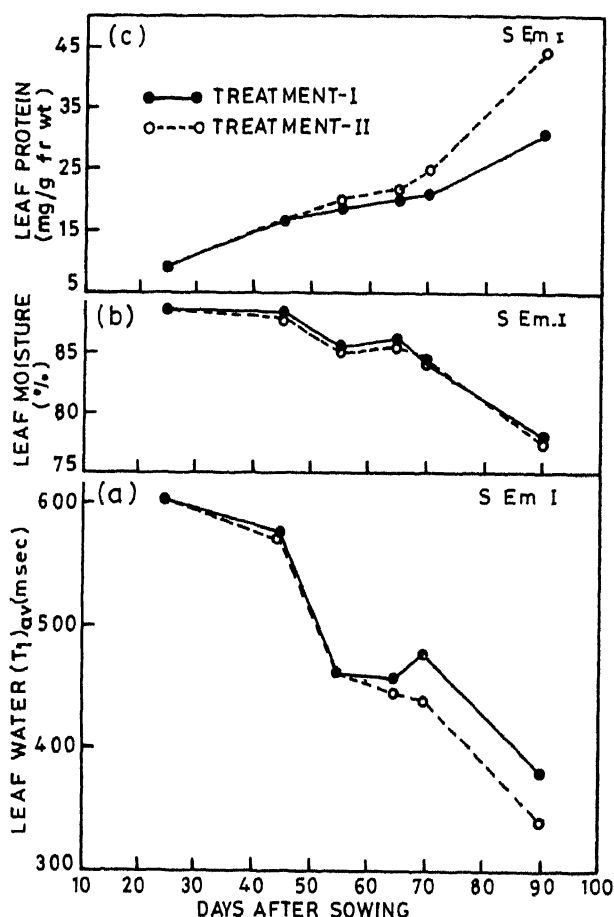


Fig 13 (a-c) Leaf water spin-lattice relaxation time T_1 , leaf moisture and leaf protein of barley variety DL120 at different stages of growth under two fertilizer treatments Solid lines represent control and dotted lines represent N-fertilized plants

Table IV
Simple linear correlation coefficients of leaf water T_1 in relation to different cell constituents

Sl No	Parameters	Correlation Coefficient
1	Leaf water content	0.676*
2	Soluble protein	-0.734**
3	Total sugar	-0.738**

*Significant at 5% level, ** Significant at 1% level

Table V
Calculated values of $T_{11(0)}$ and $T_{12(0)}$ in sec of the cell water in wheat and barley leaves

Crop Variety	A				B			
	$T_{11(0)}$	r	$T_{12(0)}$	R	$T_{11(0)}$	r	$T_{12(0)}$	r
Wheat								
C 306	1.11	0.97	0.23	0.97	0.74	0.95	0.18	0.97
IWP 72	1.25	0.90	0.33	0.89	0.83	0.97	0.25	0.94
Barley								
DL 120	1.11	0.86	0.27	0.86	0.83	0.90	0.22	0.90
Ratna	1.33	0.97	0.31	0.90	0.87	0.93	0.22	0.86

A-Calculated from the extrapolated value for zero dry matter of the plot of $R_{11}(\text{Sec}^{-1})$ and $R_{12}(\text{Sec}^{-1})$ Vs g dry wt /g water

B-Calculated from the extrapolated value for zero protein of the plot of $R_1(\text{Sec}^{-1})$ and $R_{12}(\text{Sec}^{-1})$ Vs g protein/g water

r-Correlation coefficient for R_{11} and R_{12} with g dry wt /g water and g protein/g water respectively All correlations were significant at 0.1 % level

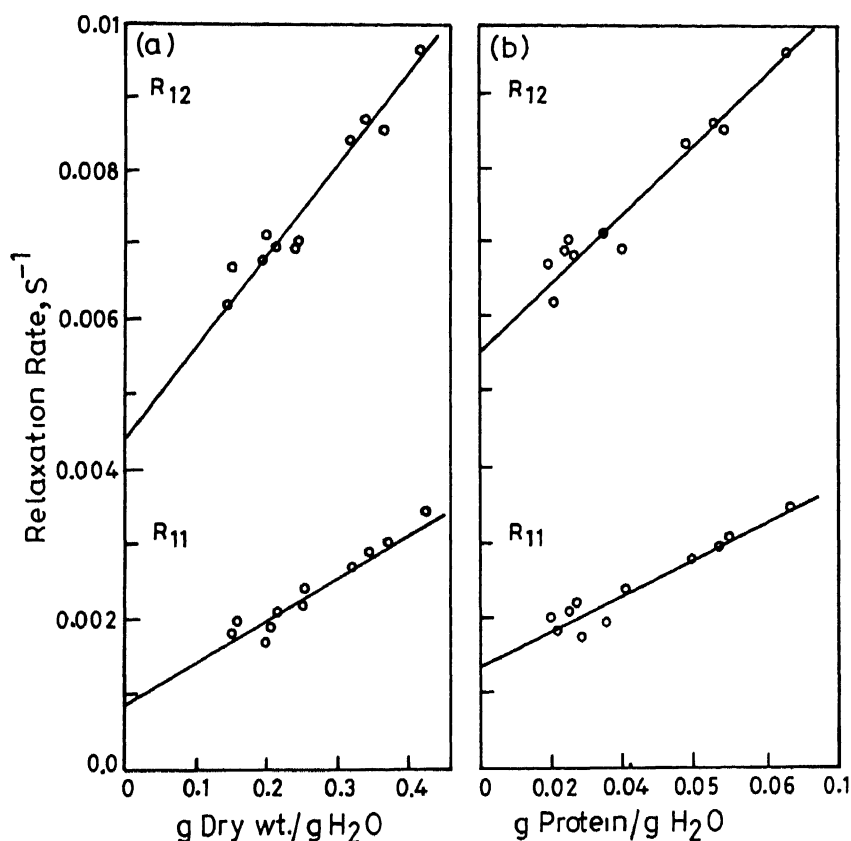


Fig 14 Longitudinal relaxation rates (R_{11} and R_{12}) of cellular water protons as a function of (a) g dry wt/g water and (b) g protein/g water in wheat leaves

perhaps due to the fast exchanges taking place in the intracellular bulk water.

2.8 Estimation of Relative Drought Tolerance in Crop Plants

Breeding for drought tolerance to increase crop production in drought prone areas world-wide is impeded by the lack of suitable screening techniques. A number of plant traits like leaf water content, relative water content, leaf water potentials, canopy temperature, osmoregulation and accumulation of certain organic compounds have been studied for the relation with drought tolerance. However, there is no general agreement on their utility as a screening parameter. Therefore, there is a need to identify a plant trait which is directly related to drought tolerance and can be measured quickly. Development of varieties tolerant to drought is an objective in many breeding programmes, but success has been limited due to non-availability of reliable and rapid screening techniques. In this laboratory, a screening method for drought tolerance has been developed based on

the measurement of leaf-water spin-lattice relaxation time T_1 of the leaf tissues.

2.8.1 Pearl Millet and Maize

In order to establish the relation between leaf water T_1 and drought tolerance, initial experiments were carried out in cereal crops which vary widely in their field tolerance to drought. Pearl millet is known to be more drought tolerant than maize. Therefore, spin-lattice and spin-spin relaxation times (T_1 and T_2) of these two crops grown in field under rainfed conditions were measured to find out the relation of T_1 and T_2 with drought tolerance²⁴. Data in Table VI clearly show that LWC, T_1 and T_2 of pearl millet were higher than those of maize which showed partial wilting whereas pearl millet did not show any such sign under the same growing condition. The difference in T_1 of the two crops was much larger than the corresponding differences in T_2 . In another experiment, T_1 and T_2 of pearl millet were found to be higher than maize even at similar LWC (Table VII) showing that higher values of T_1 and T_2 of pearl millet as

Table VI
Leaf water T_1 and T_2 of pearl millet & maize grown under rainfed conditions in field

Sample No.	Leaf water (%)	T ₁ (msec)	T ₂ (msec)	Crop condition	Degree of Drought tolerance
Pearl Millet					
1	80.2	397	86	No wilting	First
2	80.0	381	94	No wilting	
3	78.5	330	85	No wilting	
Maize					
1	72.6	194	74	Partial wilting	Second
2	73.5	193	80	Partial wilting	
3	73.0	209	74	Partial wilting	

Table VII
Leaf water T_1 and T_2 of pearl millet & maize grown in pots at three comparable levels of leaf water content

Pearl millet			Maize		
Leaf water (%)	T_1 (msec)	T_2 (msec)	Leaf water (%)	T_1 (msec)	T_2 (msec)
78.5	450	96	76.7	189	70
85.0	637	128	85.0	376	113
86.2	692	118	86.1	475	118

Table VIII
Leaf water T_{11} and T_{12} of pearl millet, barley, wheat and rice grown in pots under well watered conditions

Crop	Variety	T_{11} (msec)	T_{12} (msec)	Degree of drought tolerance
Pearl millet	ICMS 7703	850	215	First
Barley	Ratna	648	130	Second
Wheat	HD 2329	371	139	Third
Rice	Kalakari	218	53.4	Fourth

compared to maize observed in the previous experiment were not only due to higher LWC of pearl millet but the difference was inherent. In a subsequent experiment, four cereal crops namely pearl millet, barley, wheat and rice were compared for their T_1 values²⁵. The T_1 of each crop has two components (Fig. 15). The longer component T_{11} was greater by a factor of about four than the shorter component T_{12} . Both T_{11} and T_{12} differed from one crop to another. The T_{11} values of different crops were in the same order as their drought tolerance, being highest for pearl millet and lowest for rice (Table VIII).

2.8.2 Wheat

The next step in our study was to establish a relation between leaf water T_1 and drought

tolerance in a single crop species by doing experiments with the cultivars known to differ in drought tolerance. An exhaustive study was conducted for five years both in field and in pots to understand the effect of growing conditions and stage of growth on leaf water T_1 of a number of wheat genotypes varying in their tolerance to water deficit^{15,26}. In a pot culture experiment, the drought tolerant cultivars C306 and IWP-72 and the susceptible cultivar HD-2329 were grown under three different soil moisture regimes, one fully irrigated and other two under stress. The change in leaf water T_1 with maturity was monitored. The T_1 value of IWP-72 and C306 were higher than those of HD-2329 at all stages of growth and under all three moisture tensions (Fig. 16 a-c). It is apparent from these observations that the difference in T_1

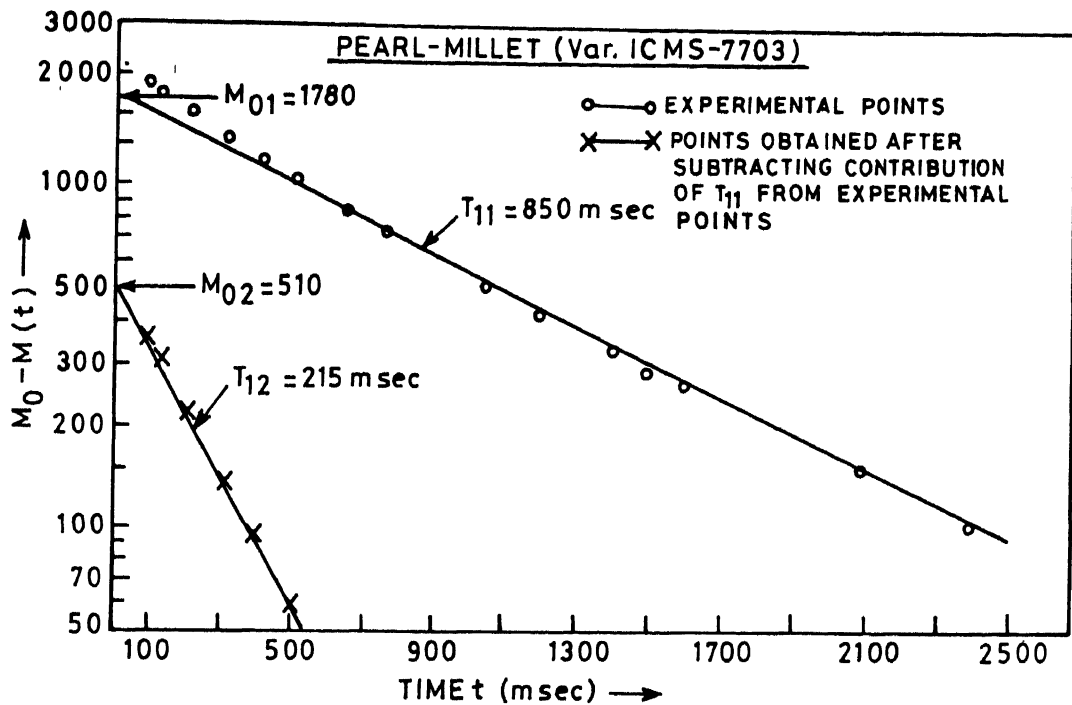


Fig 15 Semi-log plot of M_0-M_i vs time 't' for determination of leaf water T_{11} and T_{12} of pearl millet

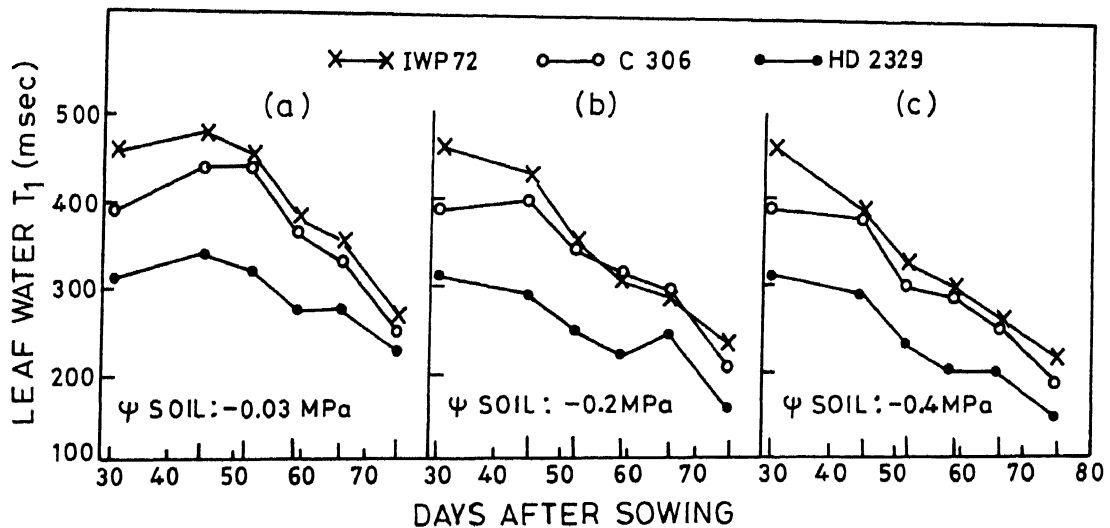


Fig 16 (a-c) Changes in leaf water T_1 with maturity of three wheat cultivars grown in pots under three soil moisture tensions (a) 0.03 Mpa, (b) 0.2 Mpa and (c) 0.4 Mpa respectively

values of the cultivars is not simply an effect of water stress but it is an inherent property of the cultivar. The change in T_1 values with maturity of six cultivars grown under irrigated condition in pots and in field are shown in Figs 17 and 18 respectively. The T_1 values decreased with maturity in pots. But in the field they increased initially up to 30 days after sowing (DAS), remained maximum between 30 to 50 DAS and decreased thereafter. The four cultivars C-306, IWP-72, WL-

410 and NI 5439 known to be relatively more drought tolerant, have higher T_1 at all stages of growth than the less tolerant cultivars HD-2009 and HD-2329. The magnitude of T_1 varied with growing conditions but the relative difference among the cultivars was maintained. The difference in T_1 was maximum during tillering stage in the field whereas the same was observed at 3-4 leaf stage in the pots. For assessing the drought tolerance of a cultivar, its T_1 should be compared

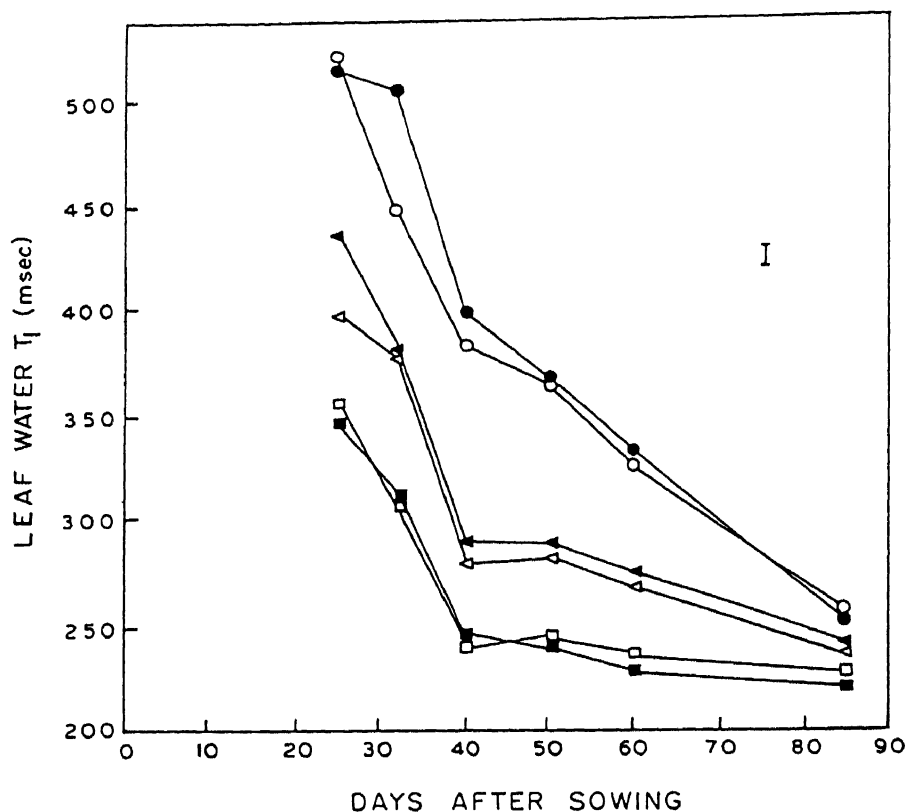


Fig 17 Changes in leaf water T_1 with maturity of six wheat cultivars grown in pots under irrigated conditions O-C 306, ●-IWP 72, ▲-WL 410, △-NI 5439, □-HD 2329 and ■-HD 2009 Vertical bars at the bottom give LSD at 5%

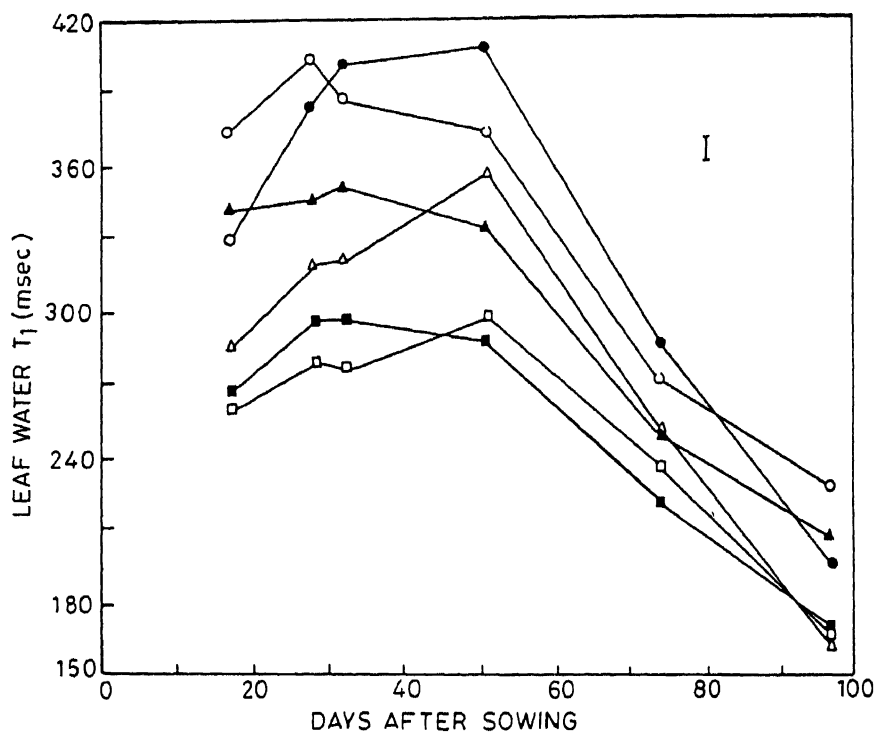


Fig 18 Changes in leaf water T_1 with maturity of six wheat cultivars grown in the field under irrigated conditions O-C 306, ●-IWP 72, ▲-WL 410, △-NI 5439, □-HD 2329 and ■-HD 2009 Vertical bars at the bottom give LSD at 5%

with T_1 of check grown under identical conditions. In a field experiment, 9 cultivars varying in their drought tolerance were grown both as irrigated and rainfed crop and their biomass reductions due to stress were compared with T_1 measurements (Table IX). The first seven cultivars which are recommended for rainfed conditions showed consistently higher T_1 compared with the last two cultivars recommended for irrigated cultivation. These 9 cultivars with differing genetic backgrounds selected for yield and other traits are not expected to give perfect correlation between T_1

and grain yield under water stressed conditions. However, reduction in bio-mass was less in the varieties showing higher T_1 values (Table IX) which indicates that leaf water T_1 is positively related to the degree of drought tolerance in wheat genotypes. In order to verify whether leaf water T_1 can be used for screening the breeding material, 20 wheat genotypes differing in their performance under rainfed conditions were raised in the field as irrigated crop. The T_1 values of 20 genotypes belonging to 3 categories of cultivation were 225-353 ms with CD = 17 msec ($P=0.05$) (Table X).

Table IX

Spin-lattice relaxation time T_1 of leaf water at 5th leaf stage and biomass at maturity of nine wheat cultivars grown under irrigated and unirrigated conditions in the field during 1987-88

S No	Cultivars	Irrigated		Unirrigated		Reduction in biomass (%)
		T_1 (msec)	Biomass (g/m^2)	T_1 (msec)	Biomass (g/m^2)	
1	C 306	364	1000	379	650	35.0
2	K 65	383	1250	411	685	45.2
3	IWP 72	387	1100	410	675	38.6
4	Hyb 65	348	976	380	650	33.8
5	WL 410	337	935	349	605	35.3
6	PBW 65	330	1134	340	667	41.2
7	HD 2428	314	975	368	450	53.8
8	Sonalika	309	1205	299	480	60.2
9	HD 2329	292	1067	305	484	54.6

Table X

Leaf water spin-lattice relaxation time T_1 at tillering stage of wheat genotypes grown in field under irrigated condition

S No	Genotype	T_1 (msec)	Recommended for
1	Mukta	342	Rainfed
2	C-306	334	-do-
3	K 65	324	-do-
4	K 8027	319	-do-
5	PBW 65	306	-do-
6	VL 616	300	-do-
7	PBW 175	293	-do-
8	IWP 72	353	-do-
9	DL 245-5	353	-do-
10	DL-153-2	319	-do-
11	WL 410	303	Rainfed & irrigated
12	HD-2385	291	-do-
13	NI 5439	284	-do-
14	Hyb 65	325	-do-
15	CPAN 1796	296	-do-
16	Sonalika	260	Irrigated
17	HD 2285	284	-do-
18	WH 147	267	-do-
19	HD 2009	238	-do-
20	HD 2329	225	-do-

The T_1 of former two groups of varieties were higher than that of the latter group. Analysis of variance of the data showed that the 3 groups of varieties differed significantly in their T_1 values.

From these studies it can be concluded that

(i) the inherent drought tolerance of wheat genotype is reflected in its leaf water spin-lattice relaxation time, T_1 .

(ii) the relative T_1 values of the genotypes are independent of growing conditions, i.e. whether grown in pots or in the field, under irrigated or unirrigated conditions.

(iii) the difference in T_1 values among the genotypes is highest when measured at tillering stage of the crop.

(iv) for assessing drought tolerance of a wheat genotype, its T_1 should be compared with T_1 of check of known tolerance grown under similar condition and measured at tillering stage.

2.8.3 Rice

Experiments were conducted with rice crop also to find out the possible relation of T_1 with drought tolerance. Extensive observations were undertaken on a large number of rice cultivars with varying tolerance to drought when grown as both direct seeded and as transplanted crop. The results can be summarized as follows,

(i) in general the drought tolerant cultivars showed higher T_1 as compared to susceptible cultivars and the difference among the cultivars was maximum at 7-8th leaf stage of the crop (Table XI).

(ii) transplanted rice showed lower T_1 compared to direct seeded rice inspite of having higher leaf moisture levels

(iii) there was an increase in T_1 at flowering stage of the crop which was more in susceptible cultivars which were generally of long duration. The large difference in duration of tolerant and susceptible cultivars was creating difficulty in comparing T_1 of the cultivars for assessing their drought tolerance

(iv) the increase in T_1 at flowering stage and the relatively lower values of T_1 for the transplanted rice could not be explained on the basis of soluble protein, soluble sugar, leaf moisture or paramagnetic ion contents.

2.9 Mechanism of Drought Tolerance

In excised leaves of three Kharif cereals widely differing in their drought tolerance, the changes with dehydration in LWC, its distribution in mobile and bound fractions in the cell water and their corresponding relaxation times were studied to understand their relation with the intrinsic tolerance to drought²⁷. The mobile fraction of cellular water declined with dehydration whereas the bound fraction remained nearly constant for certain time and then started declining (Fig. 19). This decrease in bound fraction came earlier in rice, then in sorghum and lastly in pearl millet. These results probably indicate the stage at which compartmentation of different fractions of water in the cell is lost and recovery is not possible with rehydration. Therefore, maintenance of cellular water compartmentation may be one of the

Table XI
Leaf water T_1 and leaf water content of rice cultivars grown in pots at different stages of growth

Cultivars	3rd Leaf		5th Leaf		Growth Stages		8th Leaf		Flag Leaf	
	T_1	LWC	T_1	LWC	7th Leaf		T_1	LWC	T_1	LWC
	(ms)	(%)	(ms)	(%)	T_1	LWC	(ms)	(%)	(ms)	(%)
					(ms)	(%)				
Dular	57	74.7	104	75.5	92	74.5	79	68.8	89	66.6
N-22	85	77.7	97	75.2	140	74.0	100	68.1	109	63.8
Lalnakanda	84	77.9	75	77.0	132	76.4	92	68.0	114	66.2
TKM-9	62	77.5	74	74.1	99	75.4	82	67.0	87	66.0
Gharbharn	68	77.5	98	76.6	139	76.8	102	71.4	123	68.4
P-312	50	74.1	77	74.1	85	72.6	75	66.4	79	67.1
Bala	47	74.5	82	75.9	81	71.8	79	66.5	77	66.0
Rasi	56	73.5	90	74.3	91	71.9	92	68.6	136	66.7
P-33	55	76.1	73	74.5	73	70.2	81	64.7	109	64.9
Ratna	58	72.8	63	73.9	59	72.9	64	66.6	102	66.1
Vijaya	60	75.2	78	75.2	69	72.5	67	68.6	111	66.8
Shakti	39	73.2	57	71.3	68	67.9	64	65.4	78	64.4

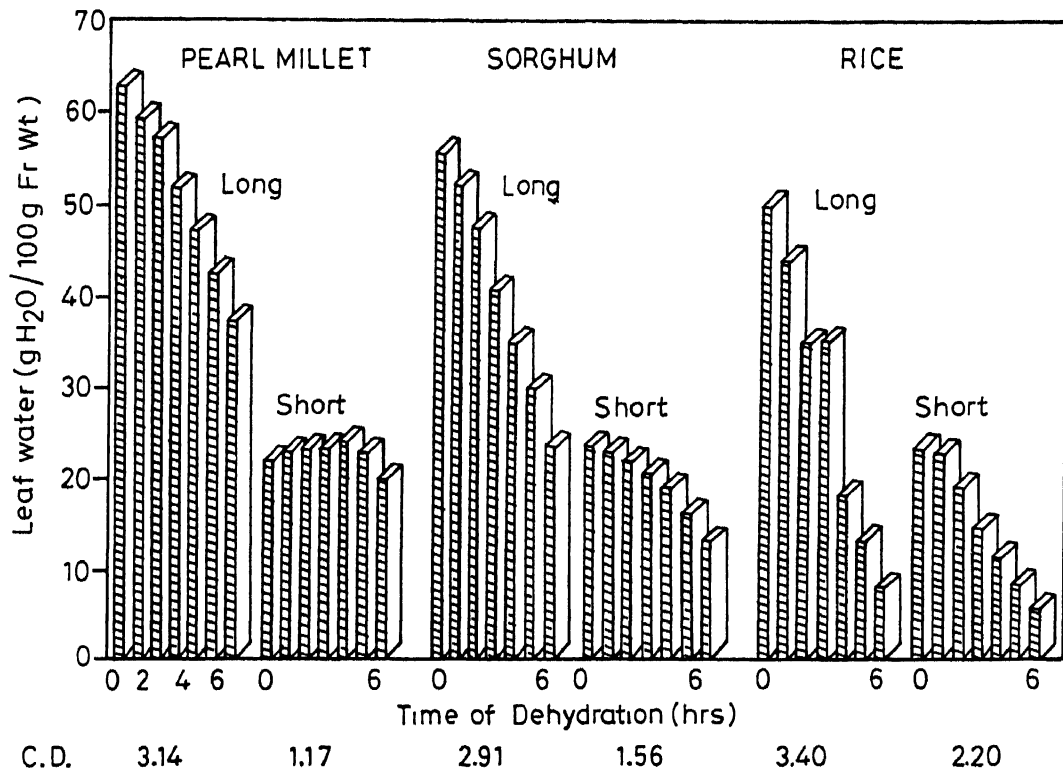


Fig 19 Changes in relative proportion of the quantity of water in the mobile and bound fraction of excised leaves during dehydration in three cereal crops

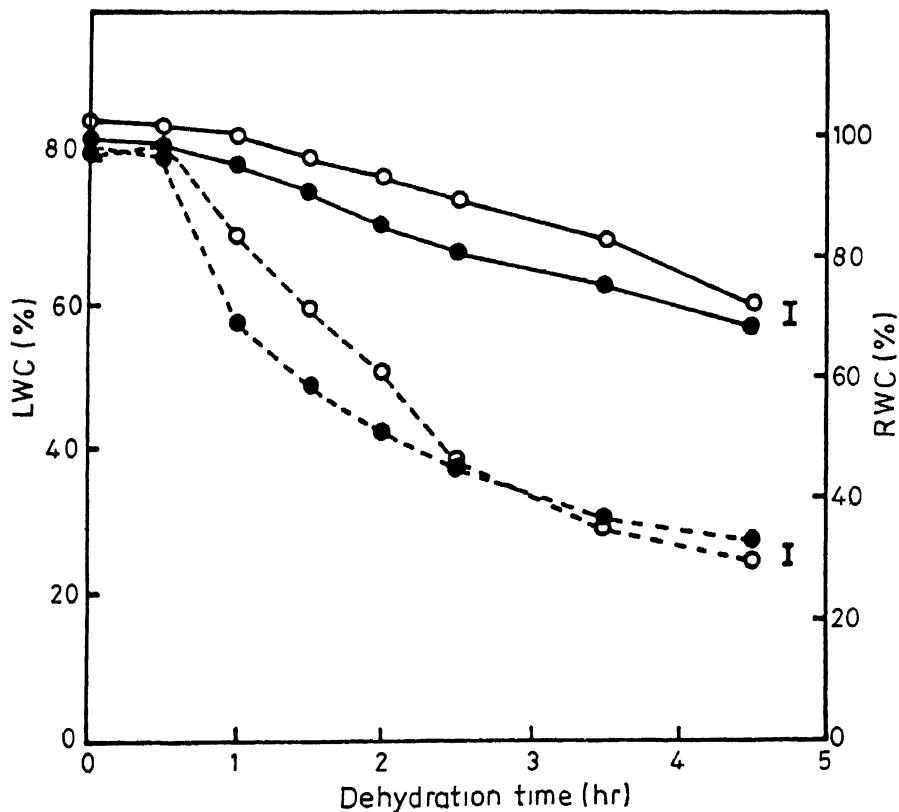


Fig 20 Changes in leaf water content (LWC) and relative water content (RWC) with time of desiccation in excised leaves of two cultivars of wheat viz, O-IWP 72 and HD 2329. Solid line for LWC and dotted line for RWC. Vertical bars indicate the average SE of the measurements.

mechanisms of drought tolerance in tolerant crop species like pearl millet.

In a similar experiment, the changes due to dehydration in LWC, RWC and proportion of cellular water in mobile and bound forms and their respective relaxation times were studied in excised leaves of two wheat cultivars which differed in their response to drought²⁸. The drought tolerant cultivar maintained higher values of LWC, RWC, T_1 and proportion of water in mobile form as compared to the susceptible cultivar during the course of dehydration (Figs 20 & 21). The differ-

ence in leaf water T_1 among the cultivars was mainly contributed by the relatively more mobile fraction of water which depleted more easily due to desiccation than the less mobile fraction

Among the Kharif cereals, there are differences in their adaptation to drought in terms of osmotic adjustment, root development and heat tolerance. In a pot experiment, plants of pearl millet, maize and rice which differ in their field tolerance to drought, were subjected to water stress and the changes in leaf water T_1 and other physiological water status parameters were monitored. All the

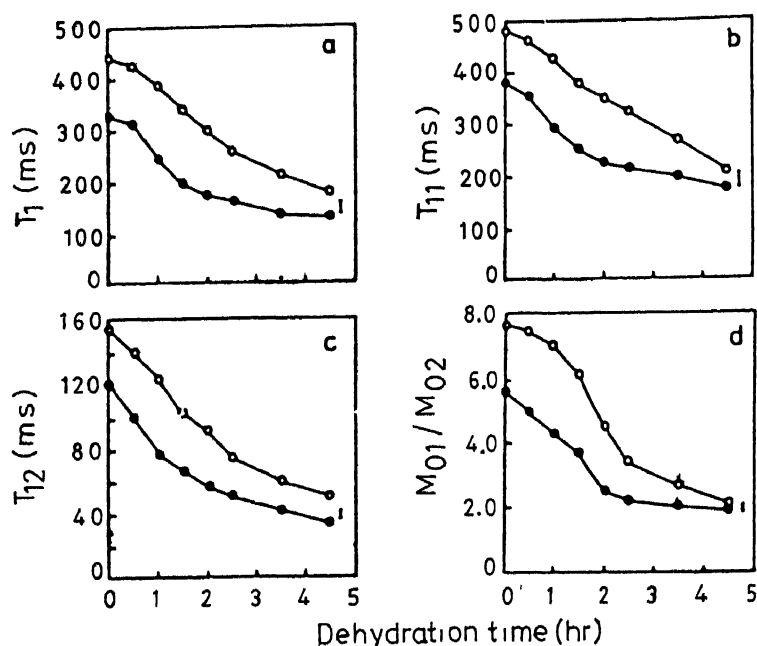


Fig 21 With desiccation time, the changes in (a) weighted average spin-lattice relaxation time of leaf water T_1 , (b) its longer component T_{11} , (c) its shorter component T_{12} and (d) the ratio of the mobile to bound fraction of cell water in excised leaves of two cultivars of wheat viz, O-IWP 72 and •-HD 2329. Vertical bars indicate the average SE of the measurements

Table XII
Variation in spin-lattice relaxation time (T_1) and other biochemical constituents in healthy and malformed tissues

Tissue	T_1 (msec)	Water Content (%)	Reducing Sugar (mg glucose equiv /g fr wt)	Protein (mg/g fr wt)
Rachis HP	275	79.3	2.3	20.8
MFP	472	80.7	5.1	8.0
Florets HP	176	76.6	2.2	19.9
MFP	267	76.5	5.5	9.9
Stem HP	221	68.3	2.3	19.9
1-2 cm MFP	354	68.6	2.4	16.9
Leaves HP	146	52.7	2.8	19.5
MFP	163	57.0	2.4	18.9
Stem HP	234	64.1	2.4	22.9
2-10 cm MFP	265	62.9	2.0	22.8

HP-healthy panicles, MFP-malformed panicles

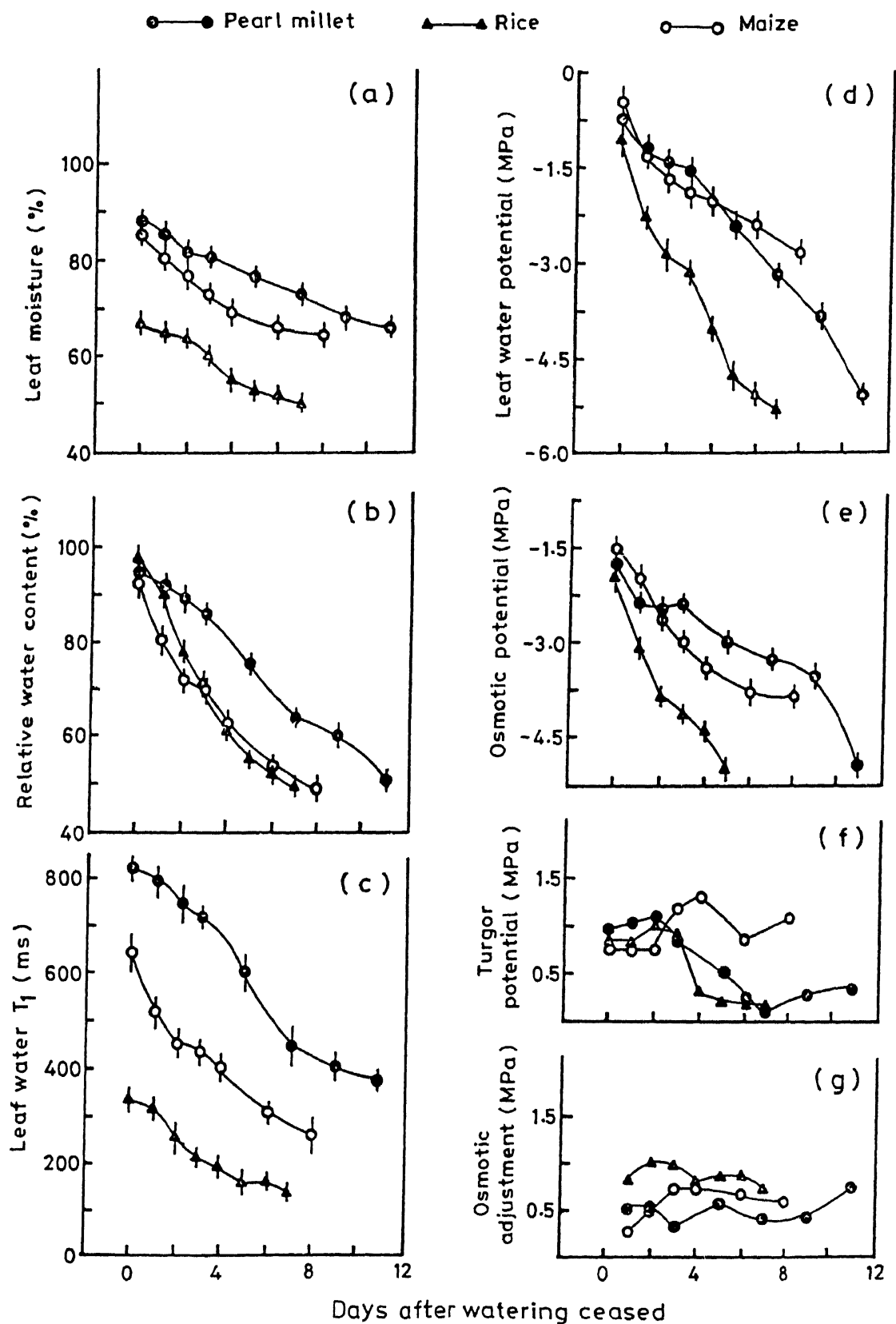


Fig 22 Changes in (a) Leaf moisture percent, (b) relative water content, (c) leaf water potential, (e) osmotic potential, (f) turgor potential and (g) osmotic adjustment in leaves of three cereals when watering was withheld for different periods. Vertical bars indicate SE of the measurements.

three crops adjusted osmotically and maintained positive turgor during stress. However, pearl millet maintained higher T_1 followed by maize and rice at all stress levels (Fig 22 a-g). Therefore, the higher drought tolerance of pearl millet could be explained on the basis of its capacity to maintain higher amounts of metabolically active water in the leaf tissues during stress cycle as compared to maize and rice²⁹.

2.10 Detection of Physiological Disorder-Floral Malformation in Mangoes

Floral malformation is a serious problem in mango cultivation as it causes heavy losses in yield and is characterized by a condensed mass of flower buds. The water status in healthy and malformed tissues in mango cv. Amarpali was studied through T_1 measurement using Bruker minispec pc-20 pulsed NMR³⁰. It was observed that T_1 values of malformed parts are relatively higher than the corresponding healthy parts (Table XII). The stem parts (1-2 cm) near the malformed panicles have significantly higher T_1 values than other parts indicating that the site of primary infection causing malformation is probably the meristematic region. The higher sugar and low protein content recorded in malformed part indicated that sugars were not being metabolized for cellular structural biosynthesis. It was further observed that the photosynthates and total solute concentration were relatively lower in malformed tissues which partly accounts for their high T_1 values. This suggests that in malformed part the disruption of normal cellular organization caused an increase in molecular

mobility resulting in higher T_1 values. The study shows the potential application of NMR for rapid assessment of malformation in mango at an early stage before the appearance of the visible symptoms.

3 High Resolution NMR

3.1 ^{13}C NMR Spectroscopy of Intact Oilseeds. A Rapid Method for Measurement of Fatty Acid Composition

The breeding of new oilseed varieties with improved quality of oil (high percentage of polyunsaturated fatty acids) while maintaining the oil yield would require characterization of the seed oil quality in a rapid and non-destructive manner. The modification of oilseed quality by conventional plant breeding or tissue culture methods would also require to analyze the variability in fatty acid composition of large number of samples of a crop. Although proton high resolution NMR spectra of oil provide well resolved resonances to facilitate measurements of oil quality (iodine values, molecular weight distribution and oil composition), but the seed spectra are very poorly resolved. However, ^{13}C NMR spectra of seeds (with chemical shift nearly 20 times larger than ^1H spectrum) provide reasonably well resolved spectra with acceptable loss of resolution due to inhomogeneities in the magnetic field. The values of T_1 's and NOE's have been measured for various resonances in seed spectra of sesame, linseed (Table XIII) and other oilseeds. The values of T_1 's, NOE's and expected line widths have been used to

Table XIII

Nuclear Overhauser Enhancement (NOE)* values for various groups of lines in seed spectra in linseed samples at 25 MHz

Chemical shift (ppm)	Sesame variety			Linseed variety		
	1	2	3	1	2	3
171.5	1.9	1.8	2.2	1.9	1.5	2.0
131.5				2.4	1.9	2.0
129.8	2.9	2.5	2.8	2.3	2.0	2.5
127.9	2.7	2.3	2.6	2.7	2.2	2.4
33.7	2.3	2.0	2.4	2.1	1.6	2.1
31.9	2.5	2.4	2.7	2.1	2.0	2.2
29.5	2.6	2.2	-	2.2	2.0	-
27.5	2.8	2.5	2.9	2.3	1.9	2.1
25.3	2.4	2.5	2.7	2.3	2.3	2.3
22.9	2.6	2.7	2.7	2.1	1.8	2.3
20.3				1.8	2.2	2.2
14.0	2.6	2.6	2.9	2.5	2.4	2.7

Reproducibility of measurement $\pm 10\%$

*Fractional increase of integrated intensity of ^{13}C resonances are irradiated

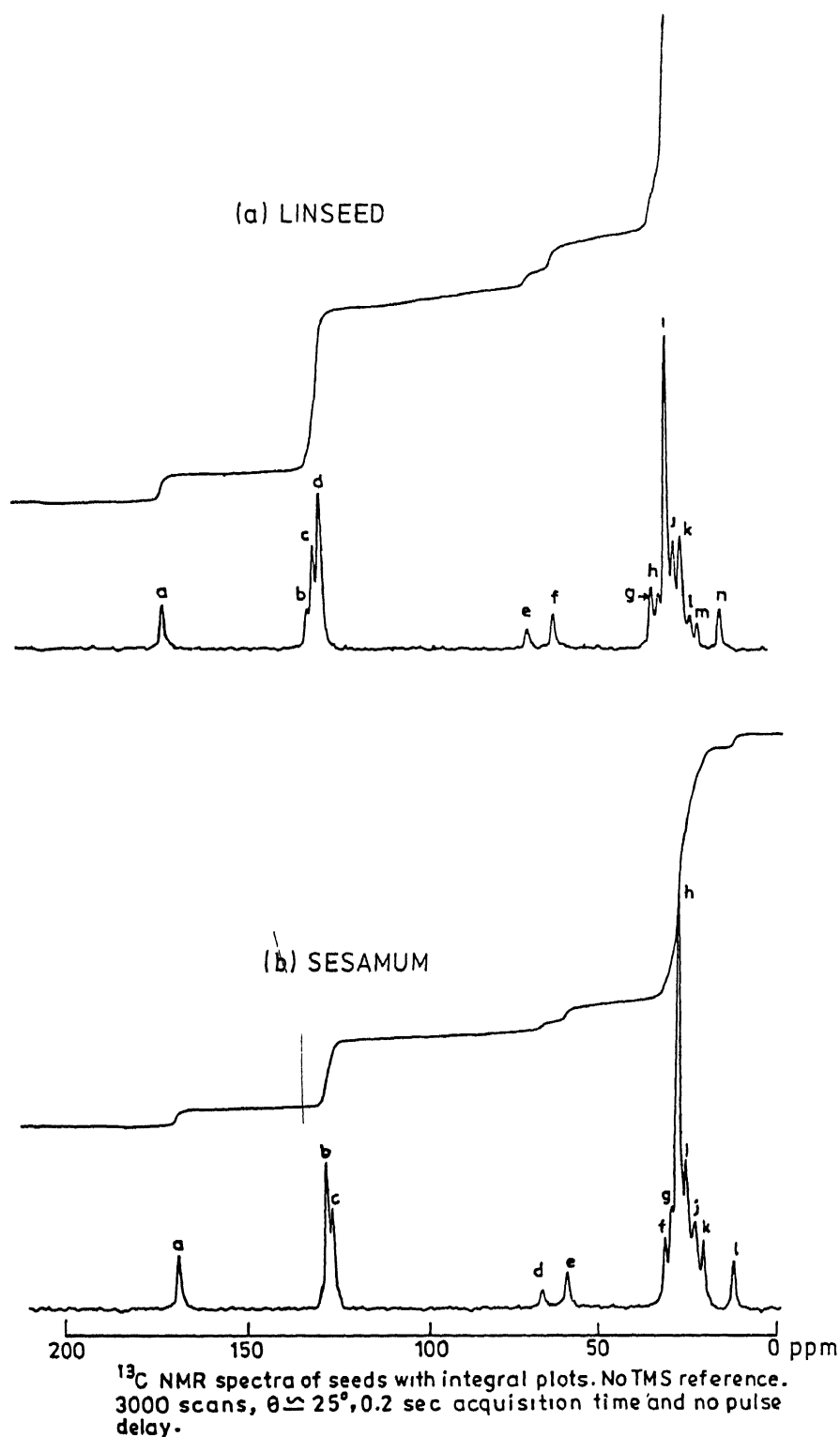


Fig 23 ^{13}C NMR spectra of (a) sesame and (b) linseed seeds using fast optimum parameters (flip angle- 25° , acq Time 0.2s, rep Time-0.2s, scans-3000)

choose the optimum parameters for taking quick spectra and selection of various peaks for computing relative proportions of oleic, linoleic and linolenic fatty acids. Using fast optimum parameters, the well resolved ^{13}C spectra of intact seeds of sesame, linseed (Fig. 23), sunflower and peanut have been obtained for rapid analysis of oil composition³¹

The fatty acid compositions of the seeds of 3 varieties each of linseed and sesame, sunflower and

Table XIV
¹³C NMR analysis of oil composition in intact seeds and comparison with GLC results (given in parentheses)

Sample	Linolenic % (18 3)	Linoleic % (18 2)	Oleic % (18 1)	Saturated % (18 0+16 0+)
Linseed 1	51.9±2.2(48.6)	12.3±2.5(8.7)	27.3±3.2(32.7)	8.5±2.5(10.0)
2	57.3±1.0(58.1)	16.1±4.3(9.5)	19.0±0.9(20.8)	7.7±2.1(11.6)
3	67.7±5.7(67.4)	11.0±3.3(9.7)	14.8±2.4(12.8)	6.5±2.0(10.1)
Sesame 1	-	38.1±1.5(39.7)	42.5±2.5(44.2)	19.4±1.7(16.1)
2	-	33.2±0.9(31.8)	47.1±3.5(50.2)	19.8±2.8(18.0)
3	-	33.0±1.4(31.6)	42.8±3.3(48.6)	24.1±2.6(19.8)
Sunflower 1	-	34.5(33.7)	48.7(54.0)	16.8(12.3)
2	-	35.3(37.8)	45.7(49.6)	19.0(12.6)
3	-	33.3(32.4)	46.1(50.8)	20.6(16.8)
Peanut 1	-	36.4(31.6)	44.0(46.3)	19.6(22.1)
2	-	34.2(29.0)	42.6(49.2)	23.2(21.8)
3	-	21.9(25.8)	46.0(50.0)	32.1(24.2)

peanut computed using integrated intensities of the peaks are given in Table XIV. Three samples of each of sesame and linseed were analyzed as three independent sets and their standard deviations are also given. The data show that the amount of saturated fatty acid calculated from the difference gives higher error to the extent of around 25 % whereas for other fatty acids it is relatively less. The individual saturated fatty acid can not be obtained by NMR method, being insensitive to chain length of constituting fatty acids to make possible the analysis of specific fatty acids. The fatty acid compositions of the same samples determined by Gas Liquid Chromatography (GLC) technique are given in the parenthesis in the Table XIV. The agreement between the NMR values and GLC values is reasonably good keeping in view the reproducibility of the former method. ¹³C NMR spectra of seeds provides a rapid and non-destructive semi-quantitative method for evaluation of oil quality in terms of individual unsaturated and total saturated fatty acids. Although the precision of measurement by this method is inferior to that obtainable by the conventional method (slow and destructive) still its potential can be utilized in large scale screening in a breeding programme for oil quality improvement. However, the precision can be improved by allowing longer analysis time.

3.2 In-vivo ³¹P NMR Study of Phosphate Uptake and Compartmentation in Maize Root Segment

The application of ³¹P NMR, an ideal technique

for studying *in vivo* metabolic mechanism in plant tissues have provided a great deal of new information which are well documented in literature. Although phosphate uptake and its intracellular distribution in root tips by ³¹P NMR are being studied since last 15 years but still the exact mechanism and various factors (energy status and effect of anoxia) regulating uptake and compartmentation of phosphate remain to be thoroughly understood.

The spectra were recorded for 25 mm long root segments excised from P-starved and P-fed plants by circulating 0.5 mM phosphate solution at pH 6.0 under different conditions.³² The aerobic phosphate loading showed (Fig 24) significant increase with time in vacuolar Pi whereas cytoplasmic Pi increased only marginally and γATP and G-6-P remained nearly constant. Using air-lift system under N₂ severe anaerobiosis could be achieved as indicated by pH drop of cytoplasmic peak. Though the cytoplasmic pH decreased from 7.6 to 7.0 during first hourly spectra under anoxia it recovered subsequently to a steady value of nearly 7.2. Under anaerobiosis (Fig 25), cytoplasmic Pi increased significantly in the absence or presence of external Pi in both P-starved and P-fed root segments, however, vacuolar Pi increased in P-starved segments only, that too in the presence of external Pi even when γATP was almost negligible. This suggests that external Pi is necessary to activate Pi carrier to transport Pi from cytoplasm to vacuole. The aerobic circulation after anoxia resulted in quick restoration of cytoplasmic pH to

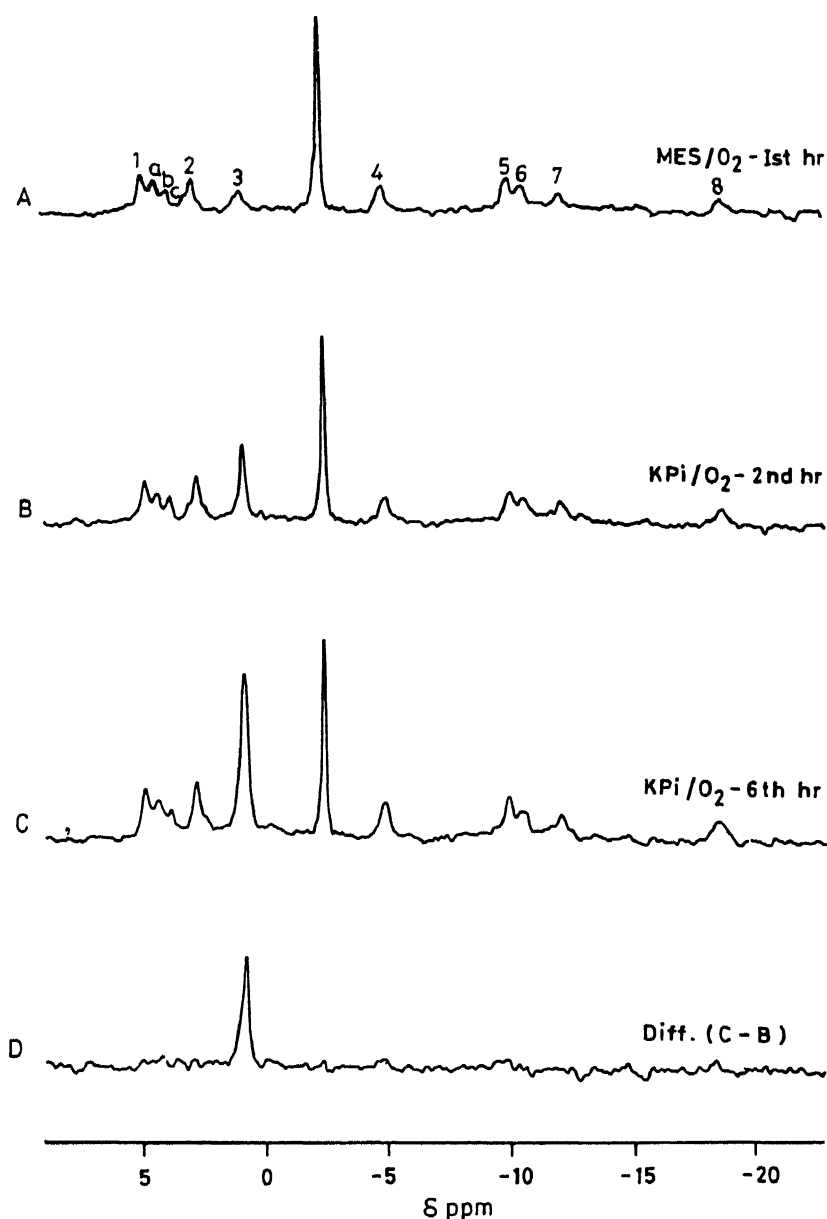


Fig 24 ^{31}P NMR spectra of 25 mm maize root segments excised from 8 days old P-starved plants (A) circulating oxygenated MES buffer of pH 6.0, (B) after one hour of replacing MES with 0.5 mM KH_2PO_4 solution under O_2 flow, (C) after five hours of circulating KH_2PO_4 solution under O_2 flow, (D) difference of spectra (B) from (C). Assignments of various resonances are 1a-Glucose 6P, 1b-Fructose 6P and other phosphomonoesters; 1c-phosphocholine; 2-cytoplasmic phosphate, 3-vacuolar phosphate, 4- γ -phosphate of NTP and β -phosphate of nucleoside diphosphate, 5- α -phosphate of NTP and nucleoside diphosphate, 6-UDPG and NAD(P) (H); 7-UDPG and 8- β -phosphate of NTP. Phosphocreatine standard signal is shown by asterisk.

preanoxic value and slow reduction of accumulated Pi in cytoplasm with accompanying small increase in vacuolar Pi.

The results indicated a strong driving force in segments excised from P-starved plants to facilitate transport of Pi across tonoplast without requiring any energy whereas in P-fed plants Pi is close to

electrochemical equilibrium and transport into vacuole may be energy requiring.

3.3 An *in-vivo* ^{31}P NMR Study of Phosphorus Metabolites in Developing Seeds of Wheat, Soyabean and Mustard

As a result of poor permeability of the layers of

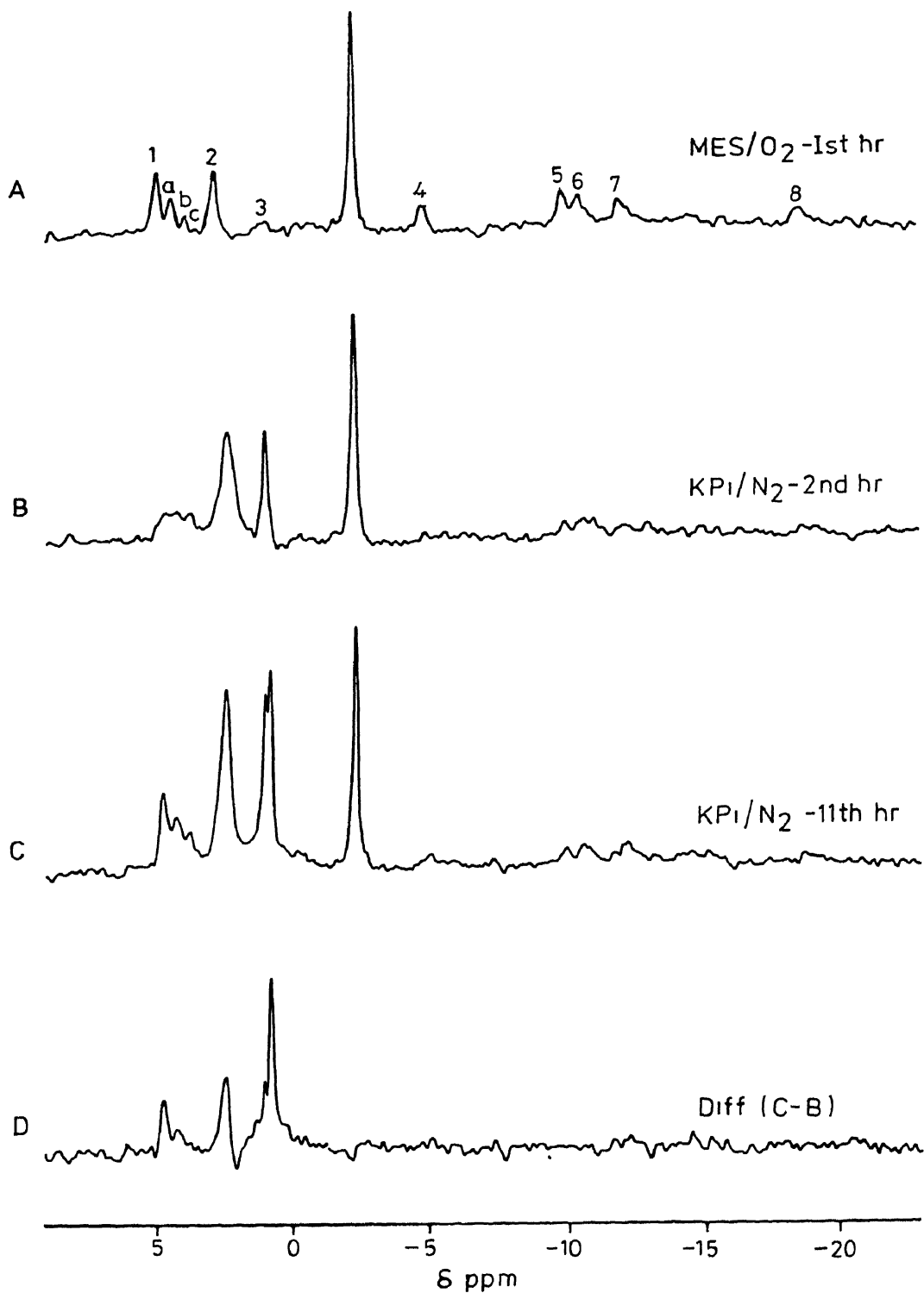


Fig 25 ^{31}P NMR spectra of 25 mm maize root segments excised from 8 days old P-starved plants. (A) First hour spectra obtained by circulating oxygenated MES buffer of pH 6.0, (B) Second hour spectra by replacing MES buffer with 0.5mM KH_2PO_4 under N_2 flow, (C) After 10 hours of circulating KH_2PO_4 under N_2 flow, (D) difference of spectra (B) from (C). Assignments of various resonances peaks are same as given in Fig 24.

the seed coat to the gaseous exchange, it is possible that access to atmospheric oxygen could become limiting for highly respiring tissues in developing seeds. This may lead to hypoxia at certain stages in development, but direct evidence for this

hypothesis is lacking. *In vivo* ^{31}P NMR spectra of maize root tips has shown that hypoxia usually leads to an acidification of the cytoplasm. This approach has been used to obtain a direct proof of existence of hypoxia in developing seeds of mustard, soyabean and wheat i.e. respectively oil, protein and starch accumulating seed-sinks³¹

In vivo ^{31}P NMR spectra of developing mustard seeds (Fig. 26) were recorded during ripening along with wheat and soyabean at 121.49 MHz using Bruker CXP NMR spectrometer. Signals were detected from phosphomonoesters, cytoplasmic and vacuolar inorganic phosphates (P_i), phytate, nucleoside triphosphate (NTP) and nucleoside diphosphosugars. Spectra of extracts showed an accumulation of phytate during development, accompanied by a decrease in P_i , and this was reflected in *in vivo* spectra (Fig. 27). The

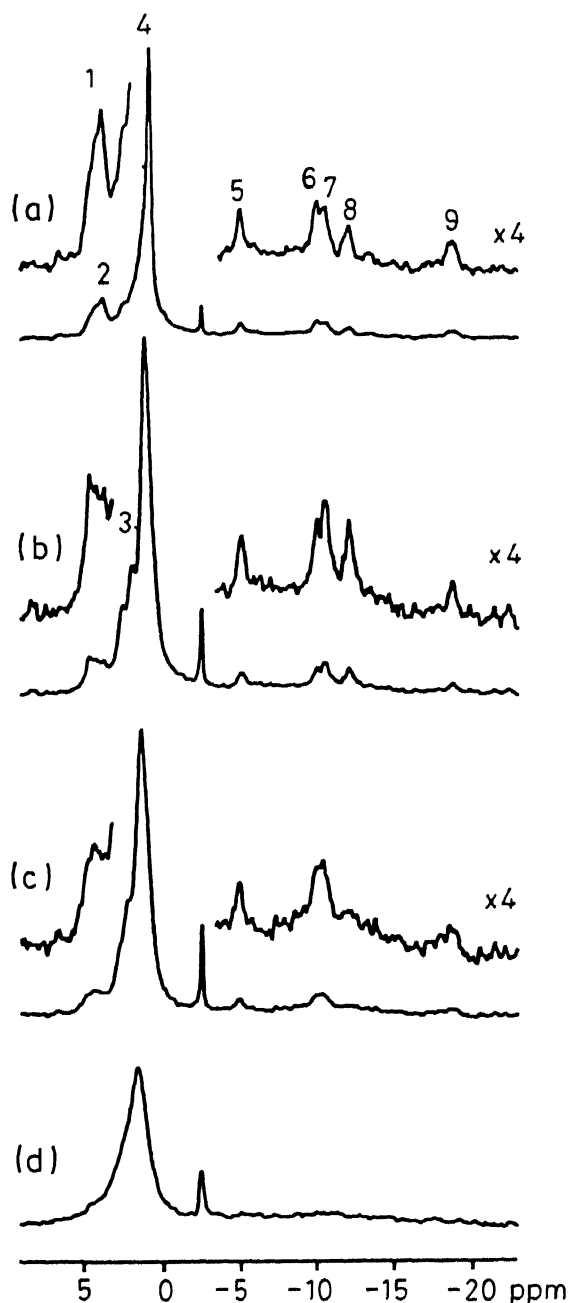


Fig 26 ^{31}P NMR spectra of intact mustard seeds (a) 14, (b) 27, (c) 40 and (d) 72 days after flowering. The numbered peaks may be assigned to 1, several phosphomonoesters, 2, cytoplasmic P_i , 3, vacuolar phytate and vacuolar P_i , 5, 6 and 9 the γ , α and β -phosphates respectively of nucleoside triphosphate, 7, UDP-glucose and NADP(H), and 8, UDP-glucose. The signal from the phosphocreatine chemical shift reference is marked with the asterisk

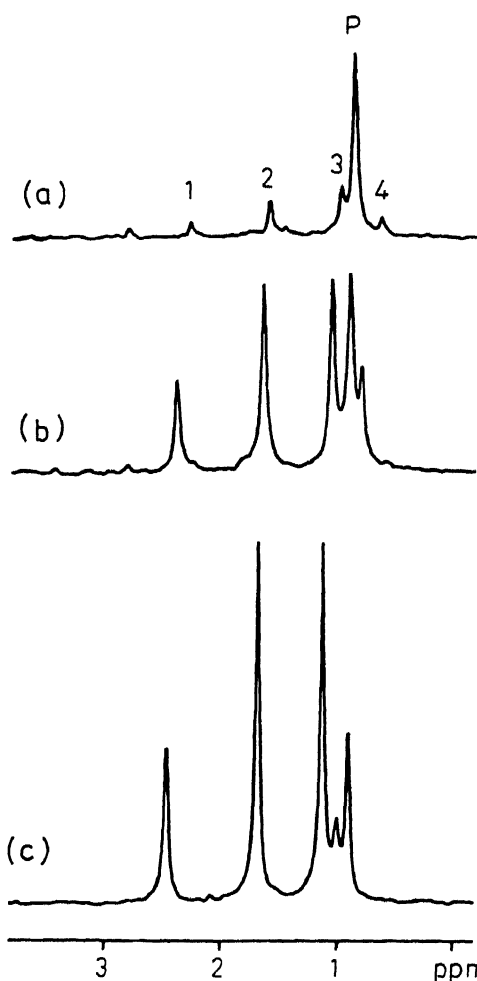


Fig 27 ^{31}P NMR spectrum of perchloric extract of soyabean seeds, (a) 20, (b) 32 and (c) 48 days after flowering (the numbered peaks assigned to phytate and the signal labelled P comes from P_i)

intrinsic width of the resonances was a significant obstacle to the interpretation of *in vivo* spectra in all the three cases, and the problem became more severe with increasing maturity. However, it was still possible to use the chemical shift of

cytoplasmic Pi signal to monitor cytoplasmic pH both in mustard and soyabean which provided first evidence of hypoxia in these two types of seeds (Fig 28). In ripening wheat seeds, chemical shift could not be ascertained due to interference from phytate signals which could not be separated from cytoplasmic Pi chemical shifts. The presence or absence of hypoxia in wheat seeds could not be ascertained in ripening seeds of wheat.

This study provides the first direct evidence of existence of hypoxic state in developing seeds and a more detailed investigation of this observation needs to be undertaken since it could have important implications for grain yield.

3.4 Osmotic Stress Induced Changes in Phosphorus Metabolism in Maize Root Segments

Many physiological and biochemical responses to osmotic stress in higher plants have been well studied but not much work has been done on *in vivo* mechanism by which plants perceive water

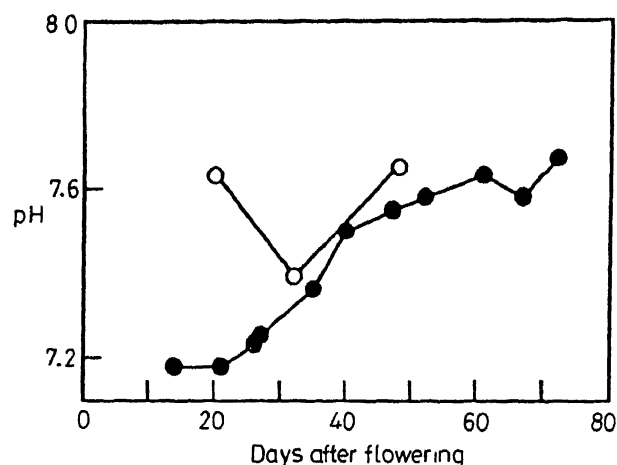


Fig 28 The cytoplasmic pH of developing mustard (●) and soyabean (○) seeds

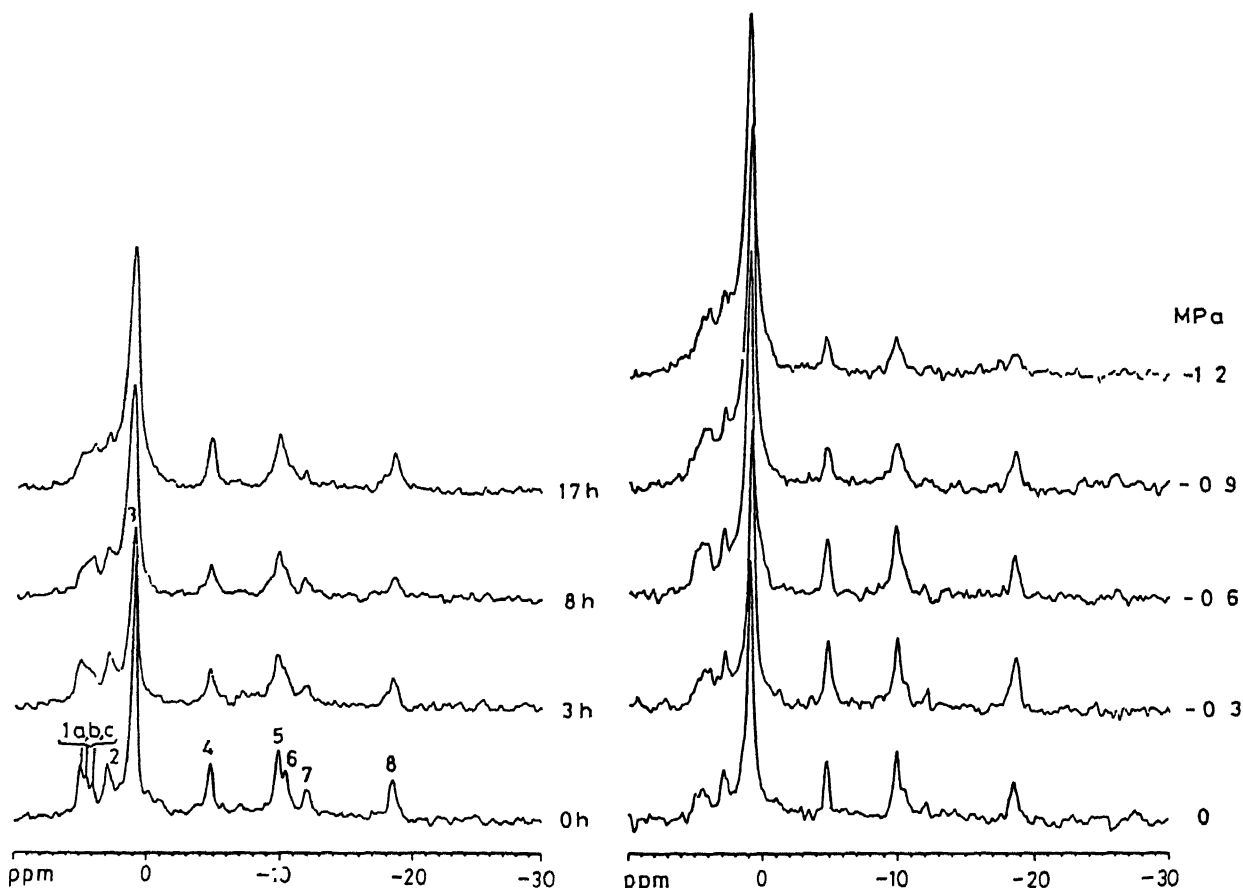


Fig 29 ^{31}P NMR spectra of maize root segments exposed to (a) hyper osmotic shock of -1.35 Mpa using PEG-6000 in the perfusion buffer and (b) gradual osmotic stress by increasing PEG-6000 in the perfusion medium. The resonance assignments are same as given in Fig 24

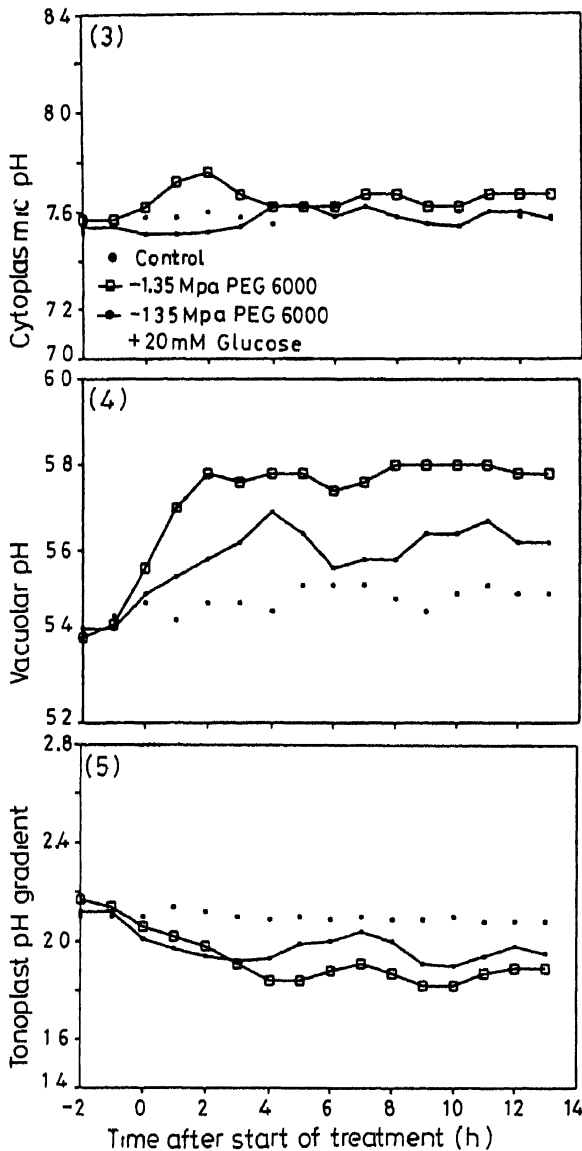


Fig 30 Time course changes in (a) cytoplasmic pH, (b) vacuolar pH and (c) pH gradient across the tonoplast of excised maize root segments as affected by -1.35 Mpa PEG-6000

stress and transduce it into a biochemical response. Therefore, a study was carried out using ^{31}P NMR to investigate the change in phosphate metabolism and intracellular pH in maize root segments to hyper osmotic shock and to compare it with that of gradual stress as it occurs in natural field conditions. The effect of sugar substrate on the adaptation of the tissue to both kinds of situations was also studied¹⁴

Due to the osmotic stress there was gradual built up of phosphocholine and decrease in glucose 6P and UDPG levels (Fig 29). The hyper osmotic shock resulted in large vacuolar alkalization and a

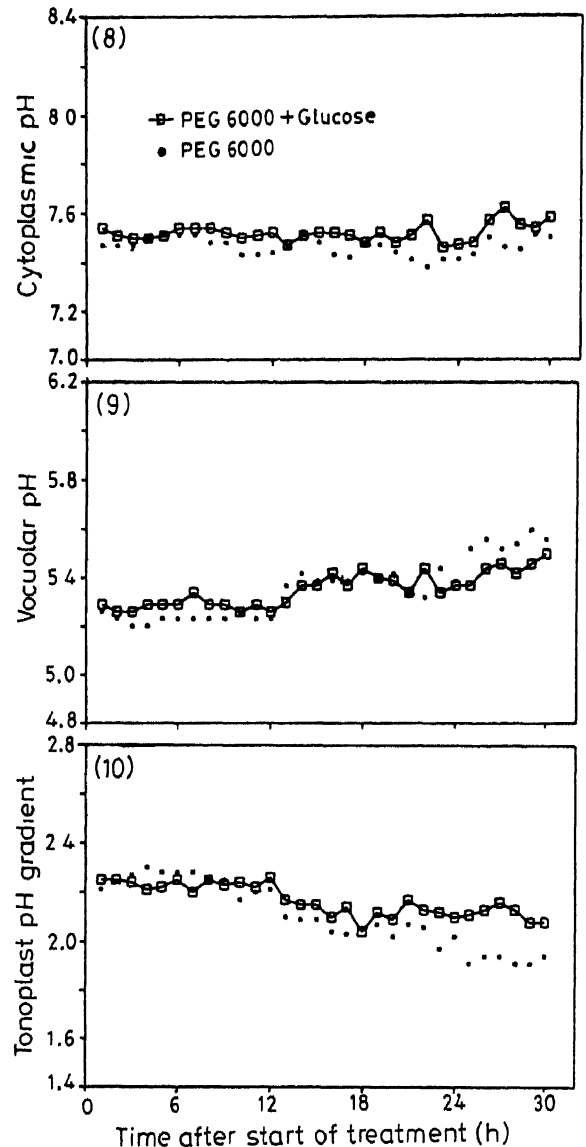


Fig 31 Time course changes in (a) cytoplasmic pH, (b) vacuolar pH and (c) pH gradient across the tonoplast of excised maize root segments as affected by gradual osmotic stress using PEG-6000

decrease in pH across tonoplast membrane (Fig. 30) On the contrary, when the stress was imposed gradually, the root segments were able to adopt to the stress and maintain pH gradient across tonoplast with marginal alkalization of the vacuole (Fig. 31). The presence of sugar in the perfusion medium reduced the impact of stress under both situations and helped the tissue to recover and adapt to the stress condition.

4 Proton NMR Imaging of Stem: A Comparison Between Pearl Millet and Maize

In the transport system of plants, stem plays a

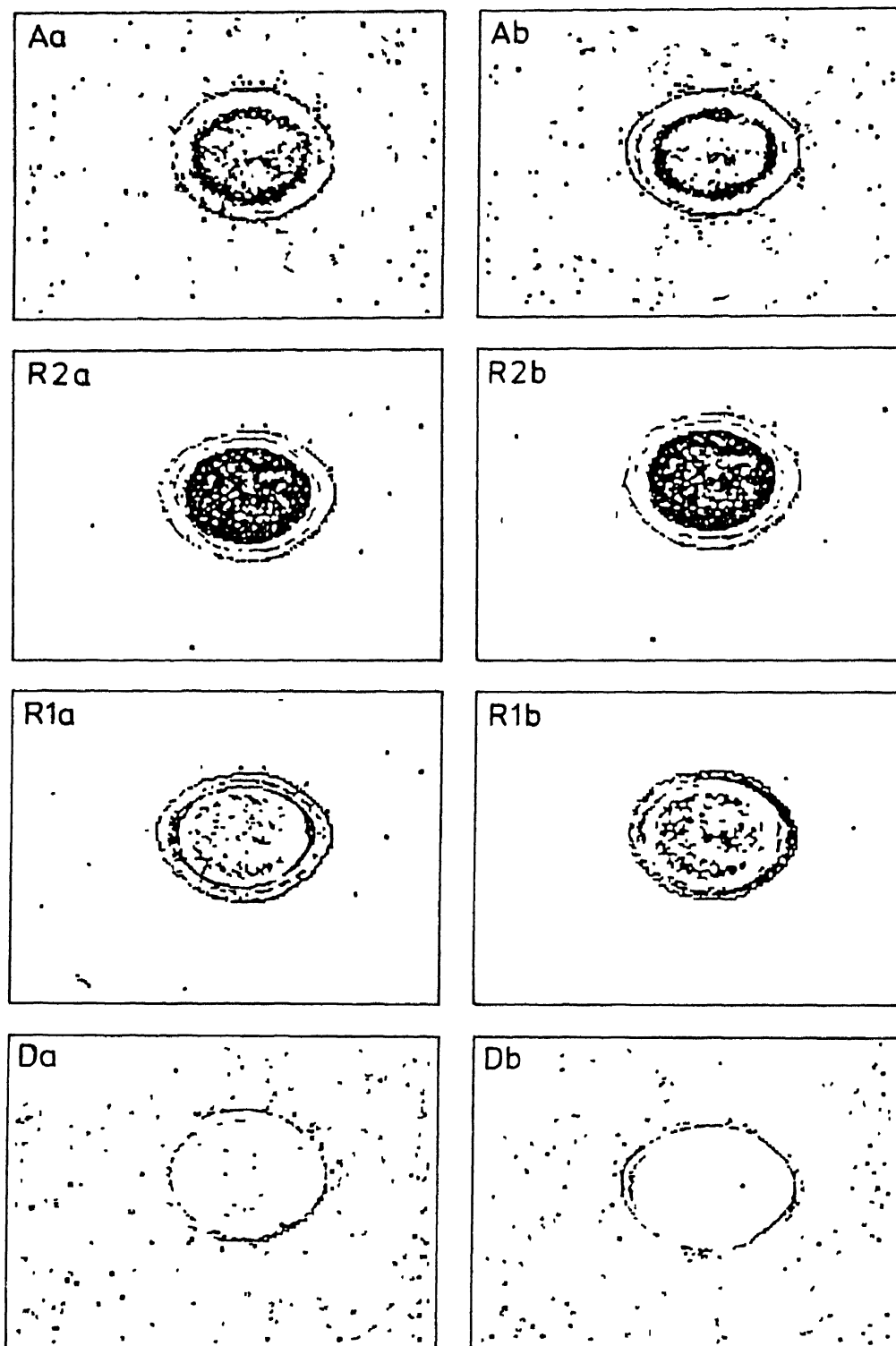


Fig 32 ^1H NMR images of month old maize stem with roots in nutrient medium or in PEG-6000 solution of -0.9 Mpa potential by inversion recovery spin echo method and pulsed gradient spin echo method A-amplitude weighted images, R2-R₂ weighted images, R1-R₁ weighted images and D-diffusion weighted images (a)-Control in nutrient solution, (b) stress in PEG-6000 solution Increase in darkness in the images indicates increase in the respective values

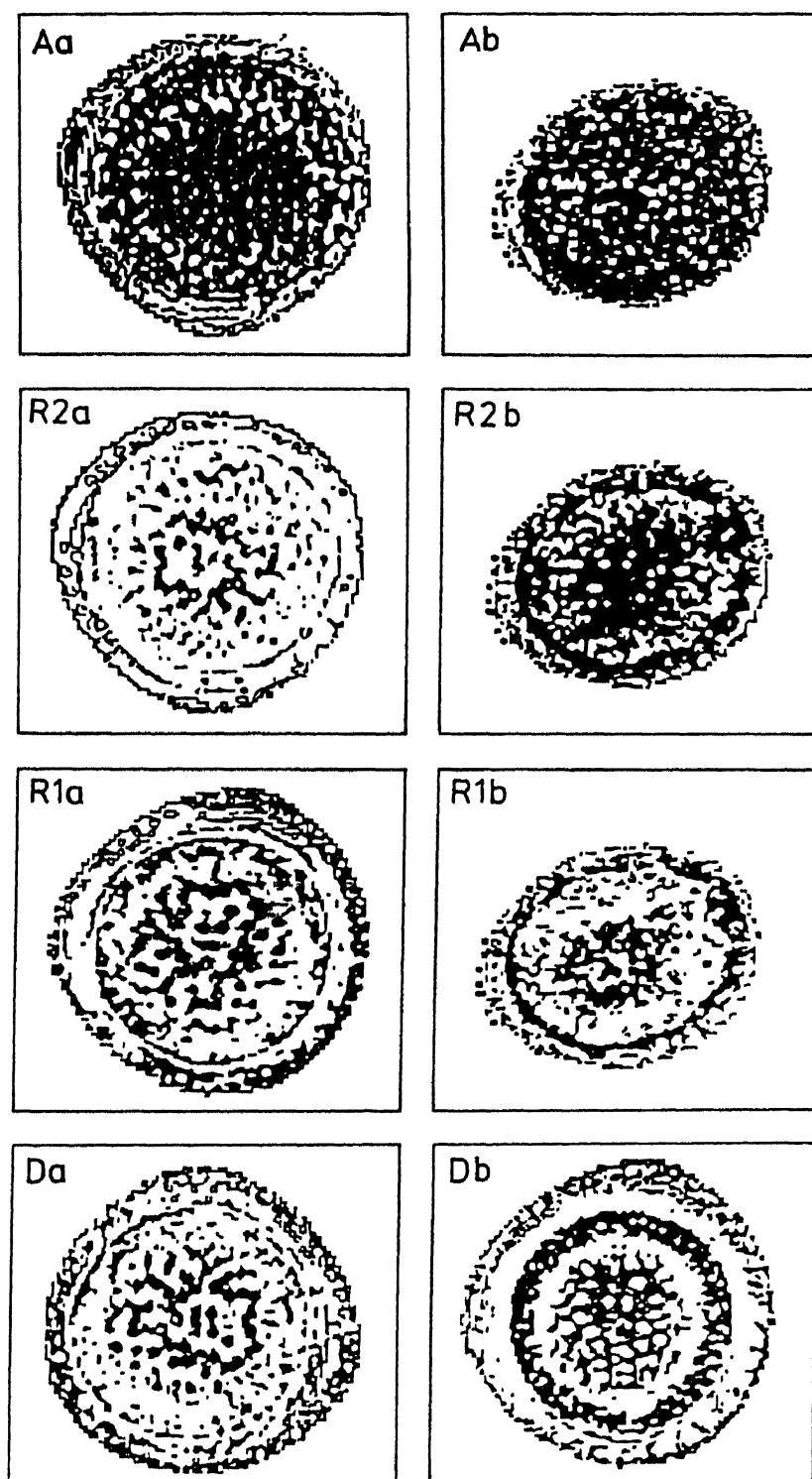


Fig. 33 ^1H NMR images of one month old pearl millet stem with roots in nutrient medium or in PEG-6000 solution of -0.9Mpa potential by inversion recovery spin echo method and pulsed gradient spin echo method A-amplitude weighted images, R2-R₂ weighted images, R1-R₁ weighted images and D-diffusion weighted images (a)-Control in nutrient solution, (b)-stress in PEG-6000 solution Increase in darkness in the images indicates increase in the respective values

major role in controlling the movement of water and in acting as a reservoir to supply to growing tissue at the time of water scarcity. NMR imaging has the potential for measuring non-invasively water movement, both self diffusion and net flow, in heterogeneous systems. When cpmg T_2 -measurements are combined with pulsed field gradient (pfg), inversion recovery (ir) and imaging experiments, proton density (amplitude), T_2 , T_1 and the (apparent) diffusion constant (D) can be studied concurrently and sequentially. This technique has been used to monitor non-invasively the water distribution and movement in stems of young plants of two cereals in response to sudden osmotic shock and subsequent recovery in nutrient medium. The images constructed by ir-cpmg and pfg-cpmg sequences for both plants are given in Figs. 32 & 33. In the susceptible crop maize, the vascular tissue dried with decrease in osmotic potential of the nutrient medium and did not support the movement of water to the growing shoot tissues. This resulted in the irreversible wilting of the plant. Pearl millet which is tolerant to osmotic stress, adapted to the stress by shrinkage of the stem region and maintained movement of water to the young leaves through diffusion. This adaptation helped in the recovery of the plant once the stress was relieved³⁵

5 Conclusion

Relaxation times T_1 and T_2 which can be easily

measured by less expensive low resolution NMR give in depth information about physical status of nuclei in its surrounding in a rapid and non-invasive manner. Like in the present report, the pulsed NMR will provide very valuable technique for developing non-invasive analytical techniques and molecular markers for rapid estimation and identification of various desirable plant traits. The non-invasive measurement of oil quality of individual seeds by ^{13}C NMR can be used as a rapid selection method in the breeding programme aimed at improving oil quality. *In vivo* ^{31}P NMR study has demonstrated the potential of this technique in understanding the mechanism of phosphate uptake and its intracellular distribution in roots, the development of hypoxia in developing seeds and the mechanism of adaptation of root segments to osmotic stress. The proton NMR imaging has been a unique technique to follow the changes in water status of the stem tissues when exposed to osmotic stress and was able to explain the adaptive mechanism of tolerant species. The present review which has covered the work of our group for the last 25 years has summarized the numerous applications of both low field and high resolution NMR in agricultural research.

6 Acknowledgements

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QUATERNARY BOROCARBIDES: THE NEWLY DISCOVERED SUPERCONDUCTORS AND THEIR NUCLEAR RESONANCE INVESTIGATIONS

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Discovery of superconductivity in the multiphase quaternary borocarbide system Y-Ni-B-C at an elevated temperature $T_c \sim 13$ K has opened up a new area of pursuit in superconductivity. Single phase quaternary borocarbide superconductors $RENi_2B_2C$ ($RE = Dy, Ho, Er, Tm$) exhibit interplay of superconductivity and long range magnetic order. Their magnetic ordering temperatures are the highest among all the known magnetic superconductors and therefore these materials are the most exciting magnetic superconductors known today. Resonance studies in borocarbides – pulsed NMR in YNi_2B_2C , Mössbauer in $ErNi_2B_2C$ & $DyNi_2B_2C$, and Mu-SR in $TmNi_2B_2C$ have been carried out. A brief summary of these results are presented in this paper.

Key Words: Quaternary Borocarbides Superconductors; Nuclear Resonance; Superconducting Critical Temperature; Antiferromagnetic Transition Temperature; Intermetallics

1 Introduction

Nuclear resonance techniques – pulsed nuclear magnetic resonance (NMR), Mossbauer effect (gamma-ray nuclear resonance absorption) and muon spin resonance/relaxation (Mu-SR) – have been extensively used to investigate superconductivity and magnetism in solids. In fact, ^{27}Al relaxation studies in aluminium metal provided the crucial experimental support to the BCS theory. More recently, important information generated by these techniques regarding superconductivity in high- T_c cuprate systems is too well known to be commented upon here.

Quaternary borocarbides are the newly discovered magnetic superconductors. Their superconducting and magnetic properties have been studied using nuclear resonance techniques. The purpose of this paper is to present a brief review of the results of these investigations. We first dwell upon the discovery of borocarbides and then summarize the results of these studies.

2 Superconducting Quaternary Borocarbides

2.1 Events That Led to the Discovery of Quaternary Superconductors

Discovery of superconductivity in the multiphase

quaternary borocarbide system Y-Ni-B-^{1,2} is among the major events in condensed matter physics that took place after the Bednorz-Muller discovery of high- T_c superconductivity. This discovery arose as a result of our interest and efforts, spanning over the last two decades, in mixed valence materials, particularly those containing nickel. As a part of this programme, we studied several members of the series of ternary borides $RENi_4B$ ($RE =$ rare earth). In samples of nominal composition of YNi_4B , we observed¹ a weak, but reproducible, signal of superconductivity at $T_c \sim 12$ K. As no binary or ternary superconducting phases of the elements, Y, Ni and B are known with such high T_c , it was clear that we had a new superconducting phase with T_c as high as 12 K in our samples. It was quite an exciting result for two reasons. (i) $T_c \sim 12$ K is high in intermetallics (not many intermetallics are known with $T_c > 10$ K) and (ii) We now have a superconducting material containing nickel metal which is a room temperature ferromagnet; not many nickel containing superconductors are known.

A minority phase stabilized by a fourth element present as an “impurity” was considered to be a possible source of the observed superconductivity.

As carbon was a likely candidate as an impurity element, samples were prepared by reacting YNi_4B and carbon together. A dramatic enhancement of the superconducting properties was observed in such materials. Also, other compositions such as $\text{YNi}_2\text{B}_3\text{C}_{0.2}$ exhibited a sizable specific heat anomaly at ~ 13 K, thereby establishing bulk superconductivity in the quaternary system Y-Ni-B-C². *This pioneering and seminal discovery^{1,2} laid down the foundation of the new and exciting field of quaternary borocarbide superconductors.* Subsequently, T_c as high as 23 K³ and two superconducting transitions with $T_c \sim 22$ K and 10 K⁴ were reported in multiphase samples of the

Pd-containing quaternary system Y-Pd-B-C. We must point out that 23 K is record high T_c in bulk intermetallics which reinforces the importance of intermetallics in search of high T_c materials

2.2 Structure of the $\text{RENi}_2\text{B}_2\text{C}$ -Phase

Fig. 1 shows a unit cell of the structure⁵ of the superconducting single phase $\text{LuNi}_2\text{B}_2\text{C}$ ($T_c \sim 16.5$ K⁶) belonging to the quaternary system RE-Ni-B-C. This is a filled-in version of the well known tetragonal ThCr_2Si_2 -structure (space group $14/\text{mmm}$) It was confirmed⁷ that $\text{YNi}_2\text{B}_2\text{C}$ and several other members of the series $\text{RENi}_2\text{B}_2\text{C}$ (RE = Ho, Er and Tm) crystallize in the same structure which is essentially a stack of infinite RE-C sheets and Ni_2B_2 ("B-Ni-B sandwiches") layers arranged in an alternating sequence along the c-axis. The short B-C bonds provide contact between RE-C planes and Ni_2B_2 -layers and are responsible for the conduction path along the c-axis. This structure reminds us of the highly anisotropic structure of the high- T_c cuprates.

2.3 Superconductivity and Magnetism in $\text{RENi}_2\text{B}_2\text{C}$

Borocarbides exhibit high T_c 's and high T_N 's, (see Table I) The exotic phenomenon of coexistence of superconductivity and magnetism occurs, in fact, at the highest ever reported temperature in borocarbides. Further still, $\text{RENi}_2\text{B}_2\text{C}$ series is unique as its members have all the conceivable combinations of T_c and T_N namely, $T_c > T_N$, $T_c \sim T_N$ and $T_c < T_N$

Several ternary superconductors RERh_4B_4 and REMo_6S_8 have high T_c and also exhibit the phenomenon of coexistence of superconductivity and magnetism for which the presence of atomic clusters in the structure of these ternaries has been regarded¹⁹ to be responsible. Borocarbides have no such clusters. Therefore, *clusters of atoms are not a crucial and essential ingredient* for high T_c and coexistence phenomena. Borocarbides, therefore, are the most exciting magnetic superconductors known so far

3 Resonance Studies in Magnetic Superconducting Borocarbides

In the remaining part of this paper, we describe briefly examples of nuclear resonance studies on some of the borocarbide superconductors. These examples will also illustrate how powerful the techniques of nuclear resonance are to get deeper

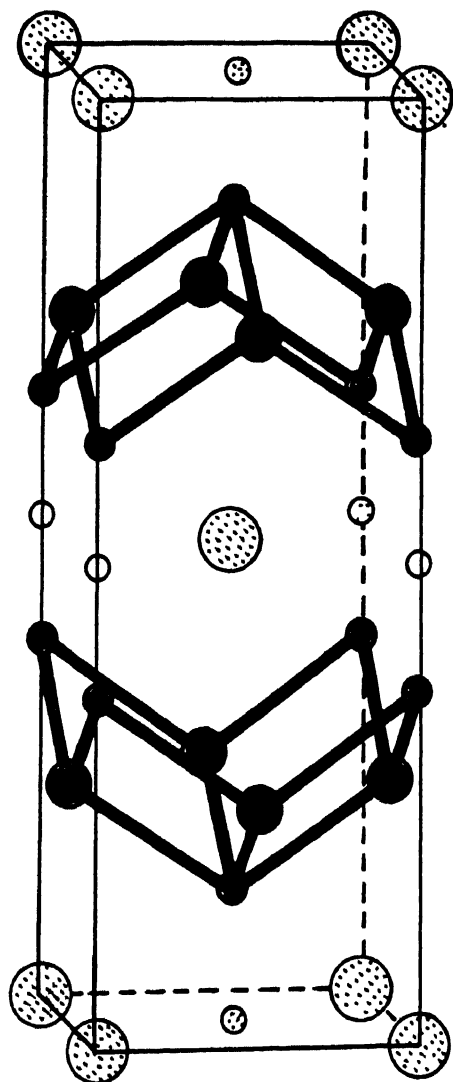


Fig. 1 Unit cell of the tetragonal ($14/\text{mmm}$) structure of $\text{RENi}_2\text{B}_2\text{C}$ materials. The bigger open circles are the rare earth atoms and smaller open circles are carbon atoms. The bigger solid circles are Ni atoms and smaller solid circles are boron atoms^{5,7}

Table I

Superconducting (T_c) and antiferromagnetic transition (T_N) temperatures in superconducting quaternary Ni-containing borocarbides $RENi_2B_2C$. Multiphase system Y-Ni-B-C exhibits $T_c \sim 13K^{1,2}$

$RENi_2B_2C$	T_c/K	T_N/K	Remarks
DyNi ₂ B ₂ C	6 ⁸⁻¹¹	11 ^{8,12}	$T_c < T_N$
HoNi ₂ B ₂ C	8 ⁶	8.5 ^{12,13}	$T_c \sim T_N$
ErNi ₂ B ₂ C	11 ⁶	6.5 ^{12,14}	$T_c > T_N$
TmNi ₂ B ₂ C	10.6 ⁶	1.5 ¹⁵⁻¹⁸	$T_c > T_N$

insight into the physical phenomena in materials of interest. The term nuclear resonance, in this paper, is used to imply nuclear magnetic resonance, Muon spin rotation/relaxation and Mössbauer resonance absorption. These three techniques have been employed in investigating superconducting/magnetic properties of borocarbides.

3.1 Nuclear Magnetic Resonance (NMR) of ^{11}B -Nuclei in YNi_2B_2C

Pulsed NMR studies²⁰ have been carried out in YNi_2B_2C both in the normal and the

superconducting state. Below T_c , one observes two resonances, one corresponding to the superconducting state and the other corresponding to the normal state. We suggested that the normal state signal arises from those regions wherein the occupancy of the B- and/or C-sites varies, at a microscopic level, from the ideal one. The relaxation rate, corresponding to the superconducting regions, decreases drastically below T_c with no enhancement just below T_c (absence of Hefel-Slichter peak). In Fig. 2 is shown the temperature dependence of $(T_1T)^{-1}$ for B-nuclei. The most remarkable feature is the rise of $(T_1T)^{-1}$ with the decrease of temperature. This suggests the presence of antiferromagnetic spin fluctuations. As no other magnetic species except Ni is present in the material, these results suggest the presence of dynamic spin fluctuations associated with Ni-derived electrons. In the Mu-SR studies on $TmNi_2B_2C$ (see below) also, we have inferred the presence of antiferromagnetic spin fluctuations. As an indirect confirmation of this suggestion, we do not observe any increase of $(T_1T)^{-1}$ in the non-

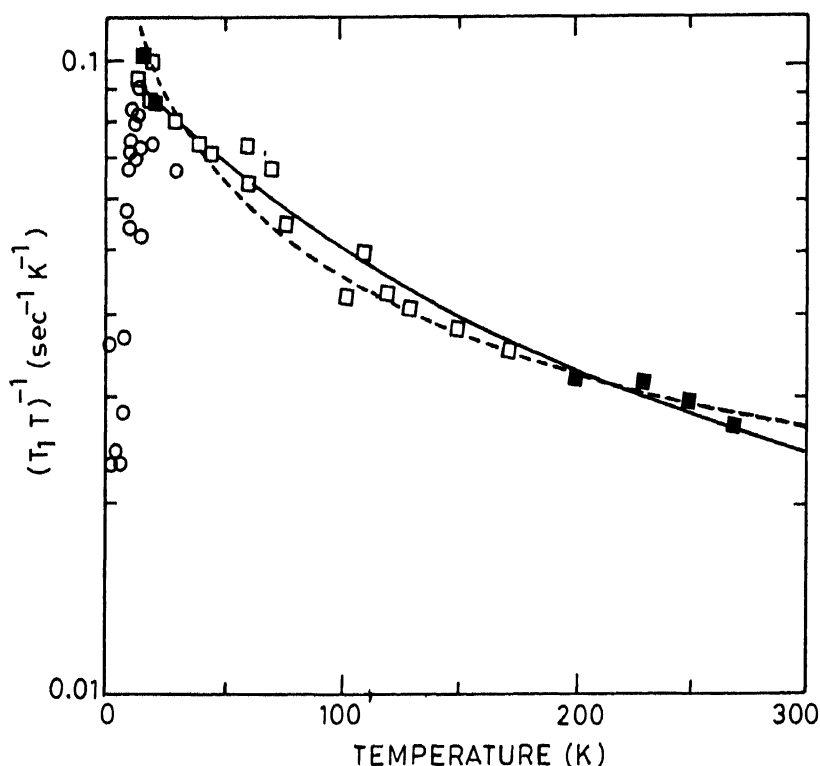


Fig. 2 Temperature dependence of $(T_1T)^{-1}$ for ^{11}B in YNi_2B_2C . Solid squares and open circles are the measured values of relaxation in the normal and superconducting states, respectively. The solid and dotted lines show the calculated values by SCR theory which correspond to $(T_1T)^{-1} = 9.86/(T + 98.1)$ and $(T_1T)^{-1} = 0.465/(T + 0.0273)^{1,2}$ for 2D-AF and 3D-AF, respectively²⁰

nickel superconducting borocarbide superconducting material $\text{LaPt}_{1.5}\text{Au}_{0.6}\text{B}_2\text{C}$. We are of the opinion that the question of (dynamic) magnetic moment associated with Ni atoms is important and is still open

3.2 Mossbauer Studies in $\text{DyNi}_2\text{B}_2\text{C}$ ($T_c \sim 6\text{K}$, $T_N \sim 11\text{K}$, $T_c < T_N$) and $\text{ErNi}_2\text{B}_2\text{C}$ ($T_c \sim 10.6\text{K}$, $T_N \sim 7\text{K}$; $T_c > T_N$)

$\text{DyNi}_2\text{B}_2\text{C}$ undergoes an antiferromagnetic ordering^{8,12} at $T_N \sim 11$ and a superconducting transition^{8,9-11} at a fairly high T_c ($\sim 6\text{K}$). It is to be stressed here that in this case, superconductivity sets in in an already magnetically ordered lattice (T_c

$< T_N$). Such materials, with $T_c < T_N$, are rather rare. Magnetic structure of $\text{DyNi}_2\text{B}_2\text{C}$, as determined from neutron diffraction studies^{16,21} consists of ferromagnetic sheets of Dy-moments (confined in the a-b plane with no component along the c-axis) antiferromagnetically coupled along the c-axis

In $\text{DyNi}_2\text{B}_2\text{C}$, ^{161}Dy -Mossbauer absorption studies²² have been carried out as a function of temperature which show that Dy-spins undergo a first order magnetic transition at $\sim 11\text{K}$, Fig 3. We shall comment further on this observation along with the Mössbauer studies in $\text{ErNi}_2\text{B}_2\text{C}$ (see below).

From the isomer shift of Dy-nuclei observed in

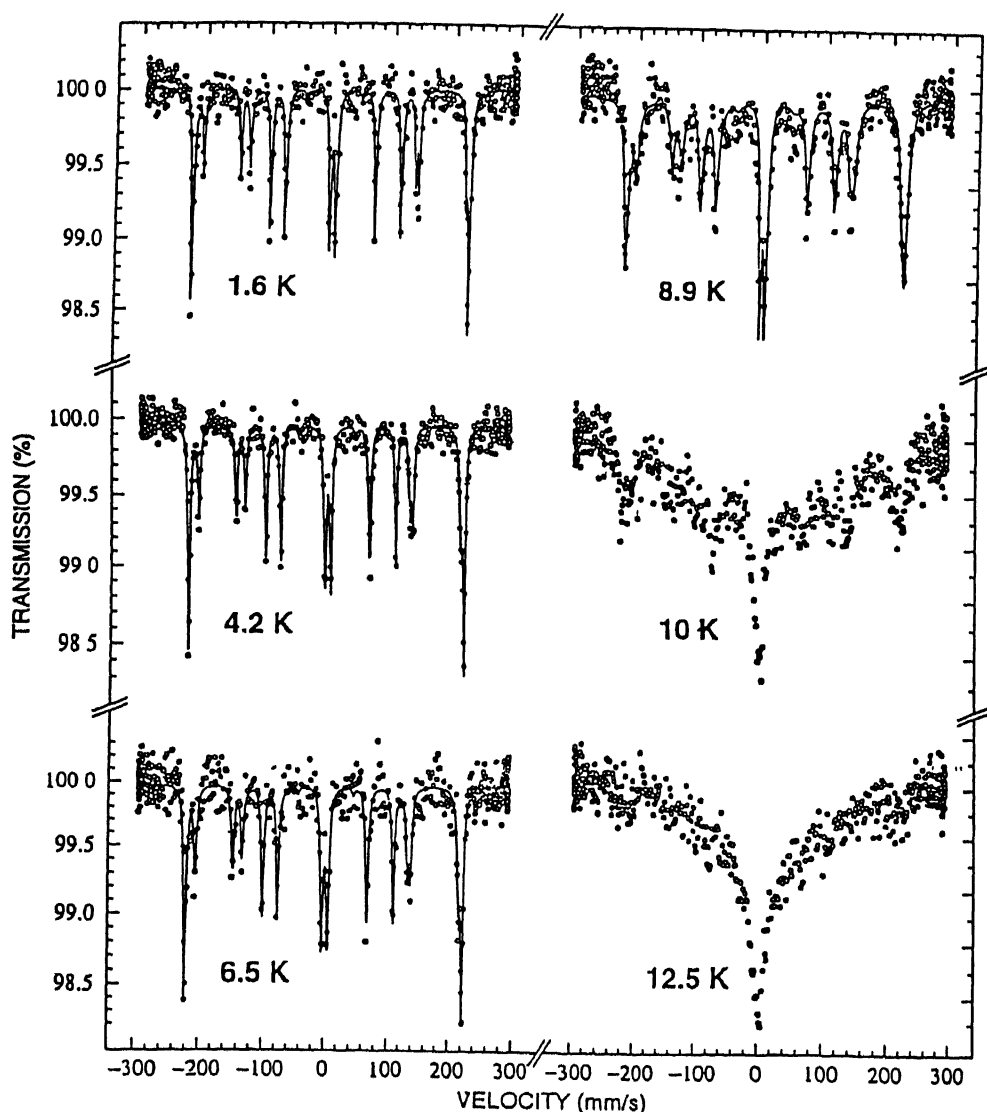


Fig 3 ^{161}Dy Mossbauer spectra of $\text{DyNi}_2\text{B}_2\text{C}$, at different temperatures. The 1.6, 4.2 and 6.5 K spectra were fitted with a "static" hyperfine Hamiltonian. The 8.9 K spectrum was fitted with a two-level exchange split relaxation model²²

this material and the systematics of the isomer shift of Dy-nucleus observed in conducting and insulating hosts, it is inferred that the Dy-C layers are insulating, and, as a consequence, Ni_2B_2 -layers are the current carriers. The structure consists of insulating and conducting sheets arranged alternately along the c-axis. It is a very important conclusion, suggesting further similarities between borocarbides and the cuprate superconductors.

With its $T_c > T_N$, $\text{ErNi}_2\text{B}_2\text{C}$ provides a complementary combination of T_c and T_N with respect to that in $\text{DyNi}_2\text{B}_2\text{C}$. Neutron diffraction studies, both in polycrystal as well as in single crystal samples, show²³ that Er-moments are ordered in a transversely polarized planar sinusoidal structure propagating along the a(b)-axis with Er-moments parallel to the b(a)-axis. No c-component was observed in these measurements.

Mössbauer studies of ^{166}Er in $\text{ErNi}_2\text{B}_2\text{C}$ ²⁴ ($T_c \sim 10.6\text{K}$, $T_N \sim 7\text{K}$; $T_c > T_N$) have shown that Er-spins undergo a first order magnetic transition at $T_N=6\text{K}$ into an incommensurate magnetic phase and the sinusoidal modulation gets fully squared up at low temperatures ($T = 1.4\text{K}$). *Er-spin relaxation rate exhibits an anomaly at $T_c = 10.6\text{K}$, Fig. 4 suggesting that the band electrons are exchange-*

coupled with the 4f-moments and are involved in the formation of the superconducting state. In fact, high T_N 's in borocarbides also imply that the RKKY-interaction is the dominant exchange interaction between the RE- and the conduction electron spins. In this respect, borocarbides are very different from the ternary superconductors, RERh_4B_4 and REM_6S_8 , wherein it is usually assumed that the superconducting electrons (derived from Rh- and Mo-metal atoms) do not have appreciable interaction with the RE-spins. More importantly, this observation raises the fundamental question about the very occurrence and survival of superconductivity in magnetic borocarbides.

In RE-based intermetallics, generally speaking, Mossbauer spectra of the RE-nuclei continue to exhibit hyperfine-splitting at temperatures much higher than the magnetic ordering temperatures. This has been understood in terms of the relaxation phenomena, viz, RE-spin relaxation time is longer than the Mossbauer measuring time. However, as we discussed above, in both cases, $\text{ErNi}_2\text{B}_2\text{C}$ and $\text{DyNi}_2\text{B}_2\text{C}$, hyperfine splitting collapses just above T_N . It is likely that the short relaxation time in these cases is due to the dynamic interaction with Ni-

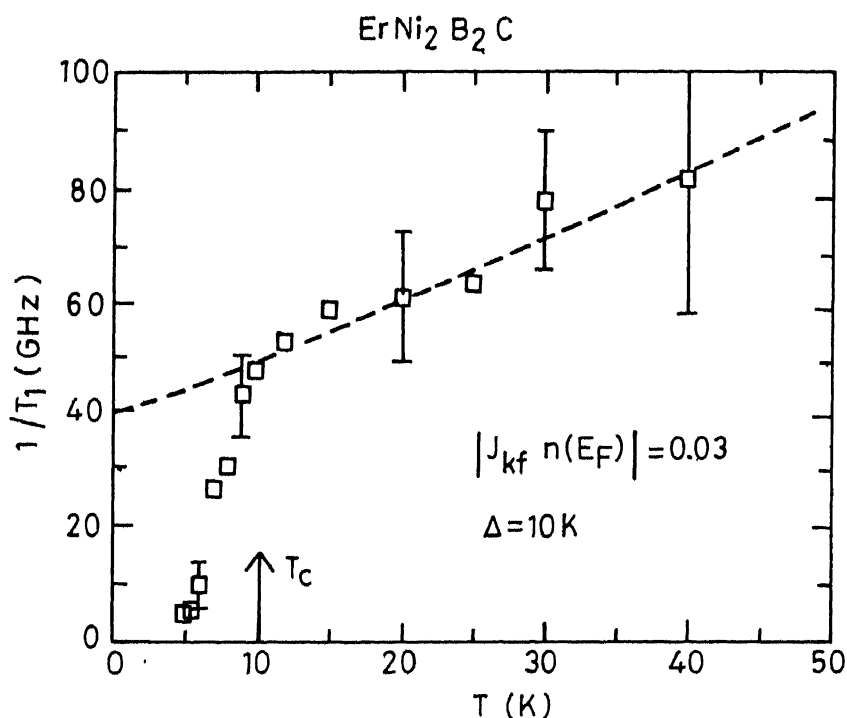


Fig. 4 Er-spin relaxation rate in $\text{ErNi}_2\text{B}_2\text{C}$. Notice a break at $\sim T_c$ ($\approx 10.5\text{K}$) in the relaxation rate. This clearly suggests that the conduction electrons interact with Er-spins and are also involved in superconductivity²⁴.

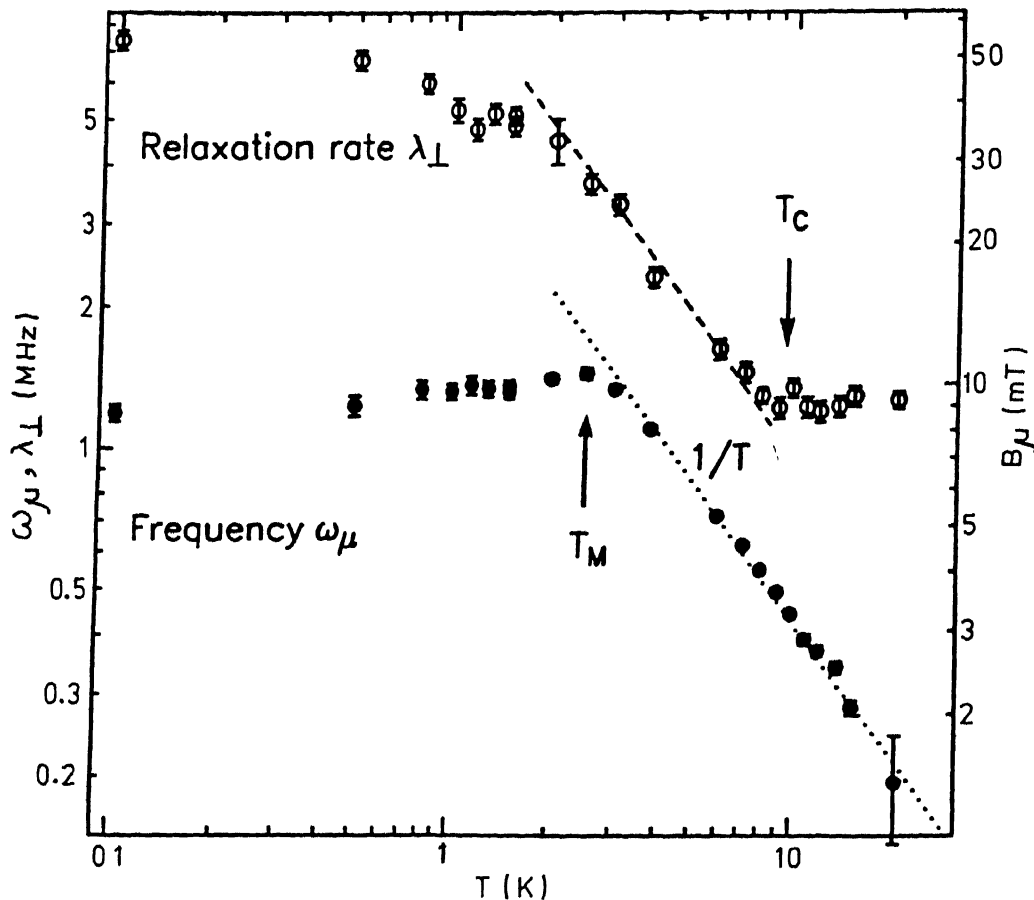


Fig. 5 Zero field μ^+ -relaxation rate (open circles) and precession frequency (filled circles) as a function of temperature for $\text{TmNi}_2\text{B}_2\text{C}$. Both data sets are plotted in MHz. The right-hand scale shows the values of magnetic field corresponding to the precession frequencies²⁵

spins which causes the collapse of the hyperfine structure above T_N . It will be highly rewarding to investigate Mössbauer spectra of several RE-based materials to check the implications of this conjecture.

3.3 $\text{TmNi}_2\text{B}_2\text{C}$ ($T_c = 11\text{ K}$, $T_N = 1.5\text{ K}$)

$\text{TmNi}_2\text{B}_2\text{C}$ has the smallest ratio $T_N/T_c = 1.5/11$ among all the magnetic superconducting quaternary borocarbides. Magnetic structure of this material is quite different from that of the other borocarbide magnetic superconductors, namely, magnetic moments are oriented along the *c*-axis¹⁶ whereas they are perpendicular to the *c*-axis in $\text{RENi}_2\text{B}_2\text{C}$ (RE = Dy, Ho and Er).

Mu-on spin resonance/relaxation (Mu-SR) measurements²⁵ have been carried out on $\text{TmNi}_2\text{B}_2\text{C}$ down to 100 mK to learn about the dynamics of Tm- and Ni-moments

Non-vanishing hyperfine field at the Mu-on site

has been observed at temperatures as high as $T \sim 50\text{ K}$ (~ 20 times higher than T_N), (Fig. 5). This implies that the Tm-spins are correlated, at the Mu-SR time scale, even at temperatures as high as 50 K. This must be contrasted with the collapse of hyperfine field just above T_N at the Dy- and Er-nuclei in $\text{DyNi}_2\text{B}_2\text{C}$ and $\text{ErNi}_2\text{B}_2\text{C}$, as observed in their Mossbauer spectroscopic studies described above

4 Summary

We have described here briefly the events that led to the discovery of superconductivity in multiphase samples of the quaternary system Y-Ni-B-C. Tetragonal structure of $\text{RENi}_2\text{B}_2\text{C}$ does not have clusters, which, as per the conventional wisdom, were considered to be essential for high T_c in intermetallics. Borocarbides have the highest T_N in the superconducting state and all possible

combinations of $T_c > T_N$, $T_c \sim T_N$ & $T_c < T_N$, making borocarbides the most exciting magnetic superconductors.

The pulsed NMR studies of $\text{YNi}_2\text{B}_2\text{C}$ suggest the possibility of fluctuating magnetic moment associated with Ni-derived 3d-electrons. Mössbauer studies $\text{ErNi}_2\text{B}_2\text{C}$ suggest that *the conduction electrons involved in the formation of the superconducting state are exchange-coupled with RE-spins*. This result is very significant and puts these materials apart from all the known magnetic superconductors. It raises some basic questions with respect to the very survival of

superconductivity under such circumstance and one is motivated to take a fresh look at the coexistence phenomena. Mu-SR studies in $\text{TmNi}_2\text{B}_2\text{C}$ point out the existence of non-zero hyperfine field at the Mu-on site even at temperatures much higher than T_N .

5 Acknowledgements

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The Collage on the Front Cover includes : A loop gap resonator (top); depiction of the nutation of magnetization under resonant pulsed excitation, the resulting free induction decay and its Fourier spectrum (bottom).

The Collage on the Back Cover includes : a set of single quantum (diagonal) and three quantum (skew diagonal) cross-section images (top) of a phantom composed of 2 tubes of ethanol and 1 of methanol - note the molecule selection and magnification/gradient attenuation in the image on the skew diagonal; ribbon diagram of the restrained minimized average NMR structure of protease-DMP323 complex (middle); proton and proton-detected carbon-13 images (bottom) of the hypocotyl region of a castor bean seedling - note the vasculature and parenchymal delineation in the water proton image at the left and the time dependent sucrose distribution in the middle and right images.

Credits

Front top and bottom . Refer to contribution by P T Manoharan and G V R Chandramouli.

Back top : N Chandrakumar and S Sendhi Velan *J Magn Reson* **A104** (1993) 115

Back middle : T Yamazaki, A P Hinck, Y-X Wang, L K Nicholson, D A Torchia, P Wingfield, S J Stahl, J D Kaufman, C H Chang, P J Dommelle and P Y S Lam *Protein Science* **5** (1996) 495. Refer to contribution by A V S Sarma and A C Kunwar

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